ANAEROBIC PSALTERIOMONAD AMOEBOFLAGELLATES

C.A.M. BROERS

ANAEROBIC PSALTERIOMONAD AMOEBOFLAGELLATES

ANAEROBIC PSALTERIOMONAD AMOEBOFLAGELLATES

een wetenschappelijke proeve
op het gebied van de Natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op
dinsdag 10 november 1992
des namiddags te 1.30 uur precies

door

CORNELIUS ADRIANUS MARIA BROERS

geboren op 30 oktober 1954
te Tilburg

Promotor : Prof. Dr. Ir. G.D. Vogels

Co-promotor: Dr. C.K. Stumm

CONTENTS

Classification of protists

Since Darwin, taxonomists have tried to classify the living organisms according to a phylogenetic system in an attempt to reflect evolution. Early biologists first divided the organisms into two main groups, the kingdoms Animalia and Plantae. In 1674 Antonie van Leeuwenhoek was the first to describe protists (11), organisms defined now as predominantly unicellular eukaryotes (12). With his hand lens "microscope" he observed and described ciliates and flagellates of which some anaerobic representatives were later identified as *Chilomastix*, *Giardia* and *Nyctotherus*. About 200 years later Haeckel recognized the protists as a separate kingdom (22). Ultimately, the increasing amount of macro- and micromorphologic data required a refinement of the traditional classification system, resulting in the widely accepted five-kingdom scheme of living organisms constructed by Whittaker (53). In this scheme the living organisms were divided into Animalia, Plantae, Fungi, Protista and Monera (= Prokaryota). The extreme diversity of the protists, however, has frustrated their classification into phyla.

During the last decade comparative studies of ribosomal DNA sequences have altered the five kingdom scheme. Not only at the highest taxonomic level has our view been dramatically changed (a tripartite division was proposed, see Table I), but also our understanding of evolutionary relationships among the protists has been greatly extended. In Fig. 1 a phylogenetic tree is shown, based on 16S-like rRNA's. In the eukaryotic rank the diplomonad flagellate *Giardia lamblia* (see also Fig. 2) represents the earliest divergence. This finding is in agreement with the primitive cellular organization of the organism and weakens the idea of derived characters as a consequence of a parasitic life style. *G. lamblia* lacks mitochondria, an endoplasmic reticulum (ER), a Golgi apparatus (14) and the 16S-like RNA has prokaryotic features (2). These characteristics make it very likely that G. lamblia diverged before the acquistion of mitochondria in the eukaryotic tree.

Also, other protists branching early from the phylogenetic tree may represent ancient phyla. These organisms are representatives of the Parabasalia (*Trichomonas vaginalis*, see Fig. 2), Euglenozoa (*Trypanosoma brucei*, *Euglena gracilis*), Heterolobosea (*Naegleria*, see Fig. 2), Acrasiomycota (*Dictyostelium discoidium*) and the amoeba *Entamoeba histolytica*. Younger phyla are ciliates and dinoflagellates, which branch off from the tree close to the

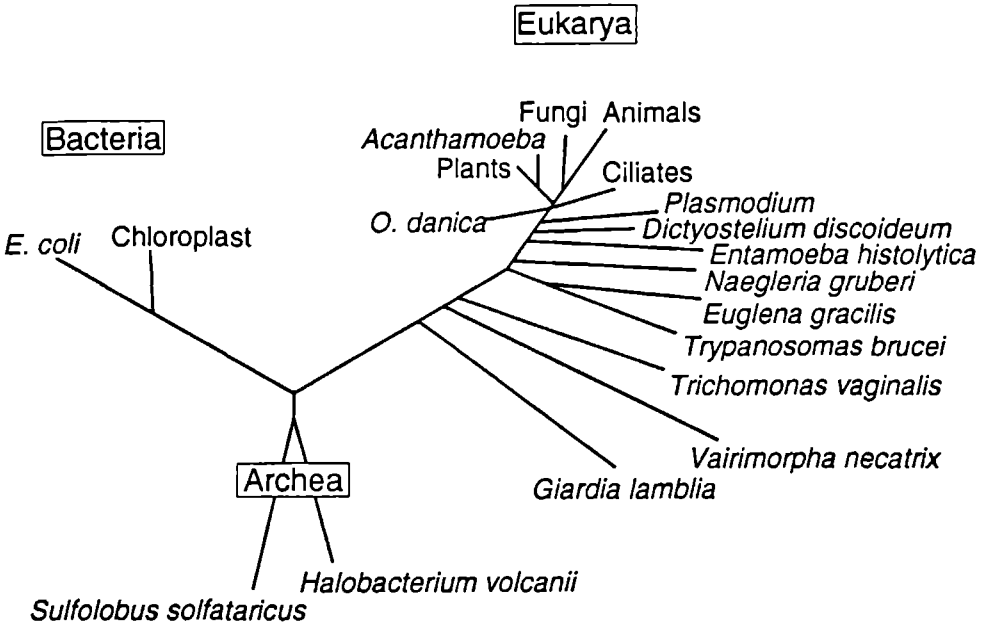


Fig. 1. Phylogenetic tree based on small subunit rRNA sequence analysis. The tree was derived by using distance matrix methods. One can distinguish three subtrees which correspond to the Eubacteria (Bacteria), Archaebacteria (Archea) and Eukaryota (Eukarya). Within the eukaryotic subtree flagellates represent the earliest divergence. Modified from reference 42.

animal-plant-fungi radiation (see arrow in Fig. 1).

From the molecular biological studies some important conclusions were drawn. Firstly, the flagellated protists have played an important role in early eukaryotic evolution (1). Secondly, amoebae have a polyphyletic origin. The emergence of nonflagellated amoeba as well as the absence of flagella in whole groups such as fungi and higher plants is a derived characteristic (1). Furthermore, the genetic diversity has led to a reconsideration of the taxonomic status of several protistan groups (7,39,41,42), with the consequence that some phyla were proposed to be combined into new kingdoms. An example is the kingdom Archezoa (4,7) which includes the phyla Archamoebae (e.g. *Mastigella*, *Pelomyxa*), the Metamonadida (e.g. *Chilomastix*, *Giardia*, *Oxymonas*) and Microsporidia (e.g. *Variomorpha*).

Table I. Classification of the living organisms according to Woese et al. (1990)

Domain (Empire)	Kingdom
1. Bacteria (Eubacteria)	
2. Archae (Archaebacteria)	
3. Eukarya (Eukaryota)	1. Animalia
	2. Plantae
	3. Fungi
	4. Protista

In parentheses: the traditional name

Eukaryotic evolution and organelle symbiosis

Because of the absence of fossil records, scenarios of the origin and evolution of the first eukaryotic cell are highly speculative or in the words of Sneath, "The history of phylogenetic studies is strewn with wrecks of broken theories" (40). However, the increasing amount of molecular, ultrastructural and biochemical data may shed some light on these early events. The amitochondrial flagellates (Archezoa) are considered as descendants of protists living in an ancient period of evolution when the earth was largely anaerobic. The change to an aerobic atmosphere forced them to adapt. According to the serial endosymbiosis theory, the first "higher" eukaryote may have arisen by the conversion of symbiotic purple bacteria into mitochondria (32,46,52). It is speculated that a quadriflagellated metamonad protist, probably resembling *Chilomastix* (Fig.2), was the host for organelle symbiosis, rather than a primitive amoeba cell (6,9,23). A predecessor of the heterobolosean amoeboflagellates is proposed to be the candidate for the first "higher" eukaryote (23). This first "metakaryote" rapidly diversified to produce a great variety of protist phyla. This process of diversification was accompanied by the development of the Golgi dictyosome and the evolution of 80S from 70S ribosomes (9,23).

The heterolobosean amoeboflagellates, two members of which are studied in this thesis, may occupy an intermediate position between the amitochondrial Archezoa and other primitive flagellates (9). Just as the Archezoa, they lack a Golgi dictyosome, which might be a primitive character (8,9). Other characteristics are a closed mitosis, mitochondria with flattened (discoid) cristae and a close association between mitochondria and rough endoplas-

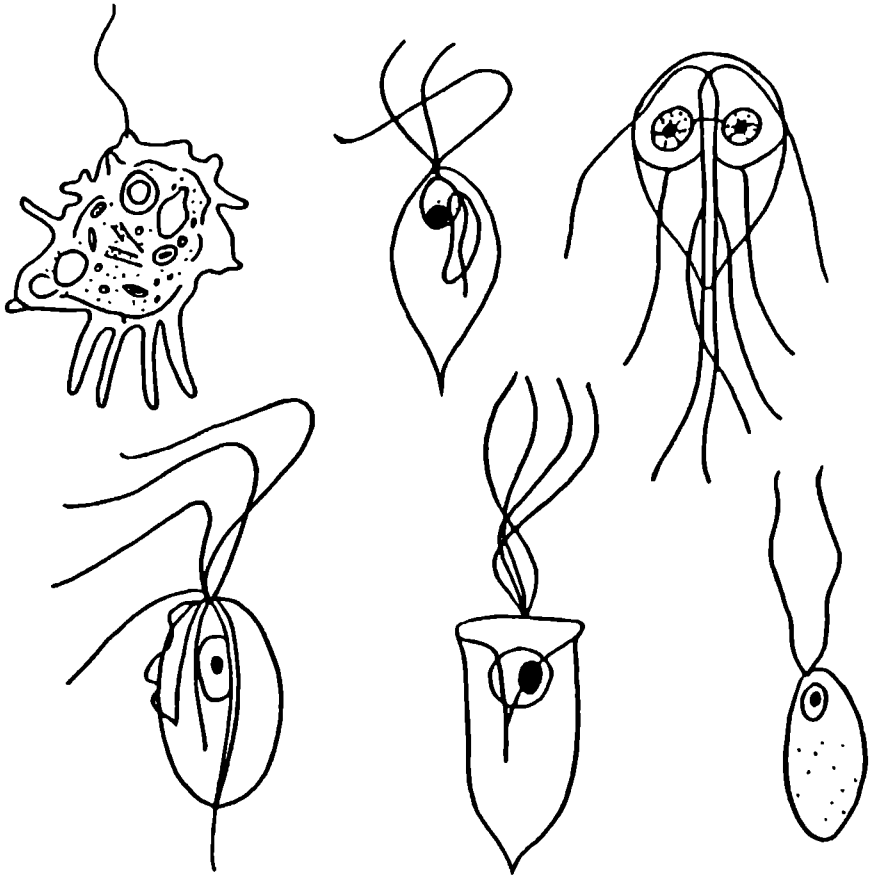


Fig. 2. Flagellated protists (a) *Mastigella* nitens after Penard (1909); (b)*Chilomastix meslini*; (c) *Giardia lamblia*; (d) *Trichomonas vaginalis*; (e) *Naegleria gruberi*; b-e redrawn after Cruickshank et al. (1973); (f) *Tetramitus rostratus* after Patterson and Zölffel (1991).

mic reticulum (35). Because of their capability to undergo a reverse transformation from amoeba to flagellates they are sometimes placed in the phylum Sarcodina (26,28,35).

Anaerobic protists

Protists are the only eukaryotes, apart from some worms, which are capable of living in anoxic environments (33,34). They perform fermentative metabolism. According to their natural habitat one can distinguish two groups of anaerobic protists: a) lumen dwellers, e.g. protists living in digestive tracts of animals; b) free-living anaerobes, e.g. protists living in aquatic sediments rich in decaying organic material. The lumen dwellers are the best-studied group and include organisms from different taxa. Some of them are parasitic, e.g. *Giardia lamblia*, *Trichomonas vaginalis* and *Entamoeba histolytica*. Many anaerobic protists lack mitochondria and consequently,oxidative phosphorylation (34). The energy metabolism of these organisms differs from their aerobic counterparts, particularly with regard to pyruvate catabolism (33). In anaerobic protists pyruvate is decarboxylated to acetyl-CoA, which is subsequently converted to acetate. This conversion yields ATP by substrate-level phosphorylation. There are two fundamentally different subcellular locations of energy metabolism in anaerobic protists (33), suggesting the following division:

1. Anaerobes with an entirely cytosolic metabolism.
2. Anaerobes in which metabolism is partly compartmentalized in hydrogenosomes. These organisms typically produce H_2.

The first group is represented by the amitochondrial Archezoa. In Fig. 3 the energy metabolism of Giardia lamblia is shown. In *Giardia* electrons generated by the decarboxylation of pyruvate are removed by the reduction of organic acceptors (29). Energy is conserved by the action of an acetate thiokinase. The major end products are ethanol, acetate and CO_2. In the second group, the conversion of pyruvate to acetate occurs in hydrogenosomes. These are membrane-bound redox organellles with a granular matrix, apparently containing no DNA (30,33,47). Hydrogenosomes were described from lumen dwelling protists as trichomonads (30,33), rumen ciliates (20, 55,56,57) and the rumen fungus *Neocallimastix* (58). The proposed metabolic pathway of the hydrogenosome is shown in Fig. 4. The reducing equivalents which are released in the oxidation of pyruvate are eliminated as hydrogen. ATP is produced in two steps by the sequential action of an acetate: succinate CoA-transferase and a succinate thiokinase.

The energy metabolism of anaerobic free-living protists, which comprise a wide variety of amoebae, flagellates and ciliates (15,45) is less well studied than that of the lumen dwellers. In these organisms, except for the Archezoan protists, organelles morphologically

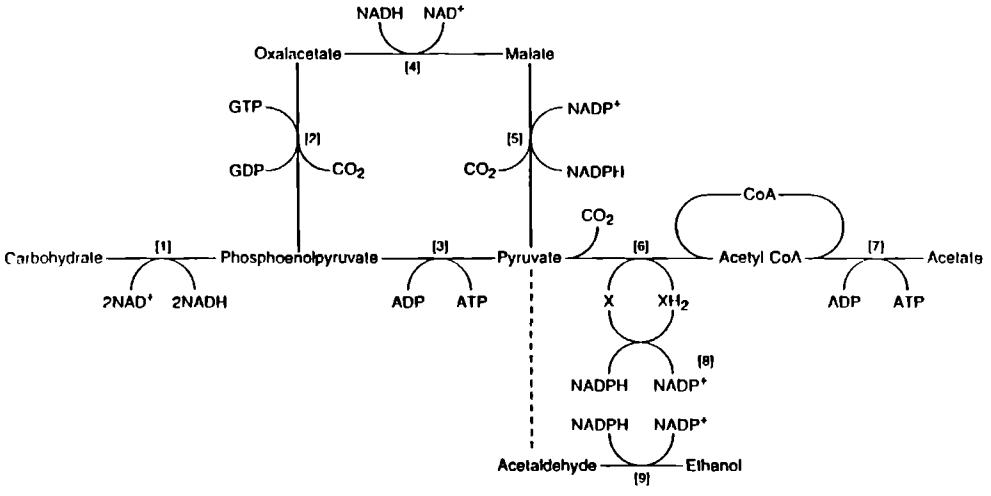


Fig. 3. Carbohydrate metabolism of *Giardia lamblia*
[1] Enzymes of the classical glycolytic pathway; [2] phosphoenolpyruvate carboxykinase (GDP); [3] pyruvate kinase (ADP); [4] malate dehydrogenase (NAD); [5] malate dehydrogenase (deacrboxylating); [6] pyruvate:ferredoxin oxidoreductase; [7] acetate thiokinase; [8] alcohol dehydrogenase (NADP); [9] NADPH oxidoreductase. Taken from reference 29.

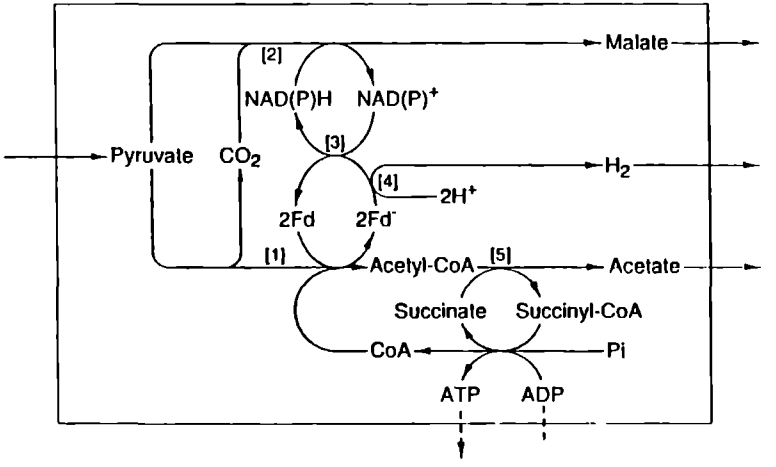


Fig. 4. Metabolic pathways of the *Trichomonas vaginalis* hydrogenosome
[1] pyruvate:ferredoxin oxidoreductase; [2] malate dehydrogenase (decarboxylating); [3] NAD:ferredoxin oxidoreductase; [4] hydrogenase; [5] acetate: succinate CoA-transferase; [6] succinate thiokinase; [7] lactate dehydrogenase; [8] phosphate acetyltransferase; [9] acetate kinase. Taken from reference 43.

resembling hydrogenosomes are found (19,44,48,49,50). In most cases these organelles are associated with methanogenic endosymbionts. These methanogenic bacteria are absent in the lumen dwellers; exceptions are the ciliate *Nyctotherus ovalis* and some trichomonad flagellates (18,27). The hydrogen requirement of the endosymbionts was an indication of the hydrogenosomal nature of the organelles (19,48,50). This was confirmed by the detection of hydrogenase, a key enzyme in the hydrogenosomal metabolism, using immuno-fluorescence and cytochemical staining techniques (3,16,59). Until now, the lack of axenic mass cultures has hampered a detailed study of the energy metabolism of free-living anaerobic protists (51). The recent establishment of an axenic culture of the ciliate *Trimyema compressum* (3) might be a breakthrough which permits advance in the knowledge of their biochemistry.

Evolutionary aspects of energy metabolism

The evolutionary origin of hydrogenosomes is enigmatic. There are two main opinions: Cavalier-Smith postulates that the hydrogenosomes are derived from mitochondria (5). Recently, this opinion was supported by some ultrastructural data (16) and by the similarities in amino acid sequence between hydrogenosomal and mitochondrial ferredoxine, as well as the presence of N-terminal signals (24). A variation on this hypothesis is the proposal that hydrogenosomes and mitochondria are derived from a common progenitor organelle (24,25). Müller (33) favours the hypothesis of an endosymbiotic origin of hydrogenosomes from anaerobic prokaryotes. The hydrogenosomal metabolism shows similarities with that of *Clostridia,* and the enzymatic composition, e.g. the presence of pyruvate:ferredoxin oxidoreductase and hydrogenase, differs fundamentally from mitochondria (33).

The anaerobic mode of life of the above-mentioned protists may represent either a primary condition or a secondary adaptation. As Archezoa are most likely primitive eukaryotes, their anaerobiosis might be a primary feature (33,34). Conversely, the hydrogenosome-containing protists have a more complex cellular organization; some of them most probably are derived from aerobic, mitochondria-containing species (5,33) as indicated by the late branching of i.e. ciliates and fungi from the phylogenetic tree (1,42) (Fig. 1). Consequently, their anaerobic mode of life might be a secondary adaptation.

Aim and outline of the thesis

Heterolobosean amoeboflagellates, e.g. *Naegleria* and *Tetramitus* (see Fig. 2), are very common in soil, fresh and marine waters (13,31). Some species of the genus Naegleria are facultative human pathogens (31). *Naegleria* spp. can be cultivated axenically (13), and progress has been made in studying their biochemistry, physiology and genetics (10,31). The flagellate stage of Naegleria possesses two flagella. In contrast to *Naegleria*, *Tetramitus rostratus* requires lower oxygen tensions (17). The flagellates of *T. rostratus* retained the ancestral tetrakonty (23) and the stability of the flagellate stage had led to the assumption of a line of descent from a *Tetramitus*-like ancestor to *Naegleria* and vahlkampfiid amoebae (38).

Until now, nothing was known about amoeboflagellates living in environments devoid of oxygen. In this thesis two anaerobic representatives are studied, belonging to the new genus *Psalteriomonas*. Both organisms were found to contain methanogenic endosymbionts. The major aim of this study was to extend the current knowledge on the endosymbiotic association between methanogenic bacteria and anaerobic protists, and to gain information which might contribute to basic evolutionary aspects of organelle symbiosis.

Chapter 2 deals with the isolation and cultivation of the anaerobic amoebo-flagellate *Psalteriomonas lanterna*. In an extensive ultrastructural study the organism is recognized as a new genus. The endosymbiotic association between methanogens and hydrogenosomes is described and evidence is given for the presence of modified mitochondria.

In Chapter 3 the effects of oxygen on *P. lanterna* are studied.

The monoxenic cultivation of *P. lanterna* and the isolation of the methanogenic endosymbiont is described in Chapter 4.

Chapter 5 deals with the characterization of the hydrogenosome-like microbodies and modified mitochondria in *P. lanterna*.

Chapter 6 reports on the cultivation of a second psalteriomonad species, Psalteriomonas vulgaris, n. spec. The isolation of its methanogenic endosymbionts and the study of its microbodies by means of indirect immunofluorescence are described.

It is difficult to establish a culture of anaerobic free-living protists. Once a polyxenic culture is established, futher study on these organisms is hampered by the relative low cell densities and by the presence of numerous food bacteria in the medium. To minimize these problems, an electromigration technique has been developed, which is described in chapter

References

1. Baroin A., Perasso R., Qu L.-H., Brugerolle G., Bachelleri J.-P. and Aouette A. (1988). Partial phylogeny of the unicellular eukaryotes based on rapid sequencing of a portion of 28S ribosomal RNA. Proc. Natl. Ac. Sci. USA *85*, 3474-3478.
2. Boothroyd J.C., Wang A., Campbell D.A. and Wang C.C. (1987). An unusually compact ribosomal DNA repeat in the protozoan *Giardia lamblia*. Nucl. Acids Res. *15*, 4065.
3. Broers C.A.M., Stumm C.K. and Vogels G.D. (1991). Axenic cultivation of the anaerobic free-living ciliate *Trimyema compressum*. J. Protozool. *38*, in press.
4. Cavalier-Smith T. (1987). The origin of eukaryotes and archaebacterial cells. Ann. NY Acad. Sci. *503*, 17-54.
5. Cavalier-Smith T. (1987). The simultaneous symbiotic origin of mitochondria, chloroplasts and microbodies. Ann. NY. Acad. Sci. *503*, 55-71.
6. Cavalier-Smith T. (1987). Eukaryotes with no mitochondria. Nature *326*, 332-333.
7. Cavalier-Smith T. (1989). Archaebacteria and Archezoa. Nature *339*, 100-101.
8. Cavalier-Smith T. (1991). Archamoebae: the ancestral eukaryotes? BioSystems 25, 25-38.
9. Cavalier-Smith T. (1991). Cell diversification in heterotrophic flagellates. In: Patterson D.J. and Larsen J. (eds.), The biology of free-living heterotrophic flagellates. Systematics Association Special Vol.45. Clarendon Press, Oxford.
10. Clark C.G. (1990). Genome structure and evolution of Naegleria and its relatives. J. Protozool. *37*, 25-65.
11. Corliss J.O. (1975). Three centuries of Protozoology: a brief tribute to its founding father: A. van Leeuwenhoek of Delft. J. Protozool. *22*, 3-7.
12. Corliss J.O. (1985). The kingdom protista and its 45 phyla. BioSystems *17*, 87-126.
13. Dexter Dyer B. (1990). Phylum Zoomastigina, Class Amebomastigota. In: Margulis L., Corliss J.O., Melkonian M. and Chapman D.J. (eds.) Handbook of Protoctista. Jones and Bartlett Publishers, Boston, pp. 186-190.
14. Feely D.E., Erlandsen S.L. and Chase D.G. (1984). In: Erlandsen S.L. and Meyer E.A. (eds.) Giardia and giardiasis. Plenum Press, New York, pp. 3-31.
15. Fenchel T. and Finlay B.J. (1991). The biology of free-living anaerobic ciliates. Eur. J. Protistol. *26*, 201-215.
16. Finlay B.J. and Fenchel T. (1989). Hydrogenosomes in some anaerobic protozoa resemble mitochondria. FEMS Microbiol. Lett. *65*, 311-314.
17. Fulton C. (1970). Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus*. In: Prescott D.M. (ed.) Methods in Cell Physiology. Academic Press, New York, pp. 341-376.
18. Gijzen H.J., Broers C.A.M., Barughare M. and Stumm C.K. (1991). Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in cockroach hindgut. Appl. Environ. Microbiol. *57*, 1630-1634.
19. Goosen N.K., Horemans A.M., Hillebrand S.J.W., Stumm C.K. and Vogels G.D. (1988). Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum*. Arch. Microbiol. *150*, 165-170
20. Grain J. (1990). Cytology and cytochemistry of rumen protozoa. Zool. Sci. *7*, 1124

(suppl.).
21. Cruickshank R., Duguid J.P., Marmion B.P. and Swain R.H.A. (1973): Medical Microbiology, Vol. 1. Churchill Livingstone, London.
22. Haeckel E. (1866). Generelle Morphologie der Organismen, Vol. 2, Reimer, Berlin.
23. Johnson A.M. (1990). Phylogeny and evolution of protozoa. Zool. Sci. 7, 179-188.
24. Johnson P.J., d'Oliveira C.E., Gorrell T.E. and Müller M. (1990). Molecular analysis of the hydrogenosomal ferredoxin of the anaerobic protist *Trichomonas vaginalis*. Proc. Natl. Acad. Sci. USA *87*, 6097-6101.
25. Lahti C.J. and Johnson P.J. (1991). *Trichomonas vaginalis* hydrogenosomal proteins are synthesized on free polyribosomes and may undergo processing upon maturation. Mol. Biochem. Parasitol. *46*, 307-310.
26. Lee J.J., Hutner S.M. and Bovee E.C. (eds.) (1985). An illustrated guide to the protozoa. Society of Protozoologists, Lawrence, Kansas.
27. Lee M.J., Schreurs P.J., Messer A.C. and Zinder S.H. (1987). Association of methanogenic bacteria with flagellated protozoa from a termite hindgut. Curr. Microbi ol. *15*, 337-341.
28. Levine N.D., Corliss J.O., Cox F.E.G., Deroux G., Grain J., Honigberg B.M., Leedale G.F., Loeblich III A.R., Lom J., Lynn D., Merinfeld E.G., Page F.C., Poljansky G., Sprague V., Vavra J. and Wallace F.G. (1980). A newly revised classification of the protozoa. J. Protozool. *27*, 37-58.
29. Lindmark D.G. (1980). Energy metabolism of the anaerobic protozoon *Giardia lamblia*. Mol. Biochem. Parasitol. *1*, 1-12.
30. Lindmark D.G., Eckenrode B.L., Halberg L.A. and Dinbergs I.D. (1989). Carbohy drate, energy and hydrogenosomal metabolism of *Tritrichomonas foetus* and *Trichomo nas vaginalis*. J. Protozool. *36*, 214-216.
31. Marciano-Cabral F. (1988). Biology of Naegleria spp. Microbiol. Rev. *52*, 114-133.
32. Margulis L. (1970). Origin of eukaryotic cells. Yale University Press, New Haven, Conn.
33. Müller M. (1988). Energy metabolism of protozoa without mitochondria. Annu. Rev. Microbiol. *42*, 465-488.
34. Müller M. (1991). Energy metabolism of anaerobic parasitic protists. In: Coombs G.H,. and North M.J. (eds.) Biochemical Protozoology 1991. Taylor and Francis, London.
35. Page F.C. and Blanton R.L. (1985). the Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica 21, 121-132.
36. Patterson D.J. and Zölffel (1991). Heterotrophic flagellates of uncertain taxonomic position. In: Patterson D.J. and Larsen J. (eds.), The Biology of Free-living Heterotro rophic Flagellates. Systematic Association Special Volume no. 43. Clarendon Press, Oxford.
37. Penard E. (1909). Sur quelques Mastigamibes des environs de Genève. Revue Suisse Zoologie 17, 407-439.
38. Robinson B.S., Christy P.E. and De Jonckheere J.F.(1989).A temporary flagellate (mastigote) stage in the vahlkampfiid amoeba *Willaertia magna* and its possible evolutio nary significance. BioSystems *23*, 75-86.
39. Schlegel M. (1991). Protist evolution and phylogeny as discerned from small subunit ribosomal RNA sequence comparisons. Eur. J. Protistol. *27*, 207-219.

40. Sneath P.H.A. (1974). Phylogeny of microorganisms. Symp. Soc. Gen. Microbiol. *24*, 1-39.
41. Sogin M.L., Gunderson J.H., Elwood H.J., Alonso R.A. and Peattie D.A. (1989). Phylogenetic meaning of the kingdom concept: an unusual ribosomal RNA from Giardia lamblia. Science *243*, 75-77.
42. Sogin M.L. (1989). Evolution of eukaryotic microorganisms and their small subunit RNAs. Am. Zool. *29*, 487-499.
43. Steinbüchel A. and Müller M. (1986). Glycerol, a metabolic end product of *Tricho monas vaginalis* and *Tritrichomonas foetus*. Mol. Biochem. Parasitol. *20*, 45-55.
44. Stumm C.K. and Zwart K.B. (1986) Symbiosis of protozoa with hydrogen-utilizing methanogens. Microbiol. Sci. *3*, 100-105.
45. Stumm C.K. and Vogels G.D. (1989). Autotrophic bacteria in protozoa. In: Schlegel H.G., Bowien B. (eds.) Autotrophic Bacteria. Science Tech Publishers, Madison, Winconsin, pp. 177-191.
46. Taylor F.J.R. (1974). Implications and extensions of the Serial Endosymbiosis Theory of the origin of eukaryotes. Taxon *23*, 229-258.
47. Turner G. and Müller M. (1983). Failure to detect extranuclear DNA in *Trichomonas vaginalis* and *Tritrichomonas foetus*. J. Parasitol. *69*, 234-236.
48. Van Bruggen J.J.A., Zwart K.B., Van Assema R.M., Stumm C.K. and Vogels G.D. (1984). *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch. Microbiol. *139*, 1-7.
49. Van Bruggen J.J.A., Zwart K.B., Hermans J.G.F., Van Hove E.M., Stumm C.K. and Vogels G.D. (1986). Isolation and characterization of *Methanoplanus endosymbiosus*, sp. nov., an endosymbiont of the marine sapropelic ciliate *Metopus contortus*. Arch. Microbiol. *144*, 367-374.
50. Van Bruggen J.J.A., Van Rens G.L.M., Geertman E.J.M., Zwart K.B., Stumm C.K. and Vogels G.D. (1988). Isolation of a methanogenic endosymbiont of the sapropelic amoeba *Pelomyxa palustris* Greef. J. Protozool. *35*, 20-23.
51. Wagener S. and Pfennig N. (1987). Monoxenic culture of the anaerobic ciliate *Trimyema compressum* Lackey. Arch. Microbiol. *149*, 4-11.
52. Whatley J.M., John P. and Whatley F.R. (1979). From extracellular to intracellular: the establishment of mitochondria and chloroplasts. Proc. R. Soc. Lond. B. *204*, 165-187.
53. Whittaker E.H. (1969). New concepts of the kingdoms of organisms. Science *163*, 150-160.
54. Woese C.R., Kandler O. and Wheelis M.L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. Proc. Natl. Acad. Sci. USA *87*, 4576-4579.
55. Yarlett N., Hann A.C., Lloyd D. and Williams A.G. (1981). Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium* Schuberg. Biochem. J. *200*, 365-372.
56. Yarlett N., Hann A.C., Lloyd D. and Williams A.G. (1983). Hydrogenosomes in a mixed isolate of *Isotricha prostoma* and *Isotricha intestinalis* from ovine rumen contents. Comp. Biochem. Phys. *74B*, 357-364.
57. Yarlett N., Coleman G.S., Williams A.G. and Lloyd D. (1984). Hydrogenosomes in known species of rumen entodiniomorphid protozoa. FEMS Microbiol. Lett. *21*, 15-19.
58. Yarlett N., Orpin C.G., Munn E.A., Yarlett N.C. and Greenwood C.A. (1986).

Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. Biochem. J. *236*, 729-739.

59. Zwart K.B., Goosen N.K., van Schijndel M.W., Broers C.A.M., Stumm C.K. and Vogels G.D. (1988). Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum*. J. Gen. Microbiol. *134*, 2165-2170.

CHAPTER 1

PSALTERIOMONAS LANTERNA GEN. NOV., SP. NOV., A FREE-LIVING AMOEBOFLAGELLATE ISOLATED FROM FRESHWATER ANAEROBIC SEDIMENTS

Europ J Protistol 25, 369–380 (1990)
June 29 1990

European Journal of
PROTISTOLOGY

Psalteriomonas lanterna gen. nov., sp. nov., a Free-living Amoeboflagellate Isolated from Freshwater Anaerobic Sediments

Cees A. M. Broers, Claudius K. Stumm and Godfried D. Vogels
Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands

Guy Brugerolle
Laboratoire de Zoologie et Protistologie, Universite de Clermont-Ferrand II, Complex Scientifique des Cezeaux, Aubiere, France

SUMMARY

A novel amoeboflagellate, isolated from anoxic sediment samples, is described and named *Psalteriomonas lanterna* gen nov , sp nov The cells of the flagellate stage show a fourfold rotational symmetry with four nuclei, four ventral grooves and four mastigont systems Each mastigont has four flagella of equal length Microtubular roots, striated roots or rhizoplasts and electron dense structures are associated with their basal bodies A Golgi apparatus is absent Organelles surrounded by rough endoplasmic reticulum (RER) are presumably modified mitochondria Methanogenic endosymbiotic bacteria are closely associated with microbodies and form a central body Nuclear division shows the characteristics of a closed mitosis Cells of the amoeboid stage are mononucleated and lack the methanogenic endosymbionts Reproduction occurs in both stages of the life cycle

Introduction

Oxygen free sediments, mainly consisting of organic material, are called sapropel [13] Sapropelic amoebae and ciliates contain methanogenic bacteria which live inside the protozoan cells [22] The methanogenic endosym bionts can be seen *in situ* by means of epifluorescence microscopy [7] During the study of the sapropelic microorganisms [23] flagellates have been rather neglected This paper reports the isolation and cultivation of a hitherto undescribed amoeboflagellate which contains methanogenic endosymbionts The organism undergoes flagellate-to amoeba transformation, suggesting a relationship to members of the Heterolobosea [18] The general morphology, the organization of the mastigont system and the ventral grooves will be described in an effort to classify the organism Some preliminary results of this study were published in the Proceeding of the Symposium on "Anaerobic Protozoa" held in Cardiff on July 23–24, 1988 as a satellite meeting of the 41st Meeting of the Society of Protozoologists at Bristol [3]

Material and Methods

The amoeboflagellate was isolated from an anoxic sediment from a sewage treatment plant near Nijmegen Subsamples were transferred to 1 litre bottles and subsequently enriched with 0 2 g casein hydrolysate and 0 2 g peptone to stimulate bacterial growth Monocultures were obtained after several weeks by transferring single flagellates with a micropipette to sterilized medium, containing 5 mM phosphate buffer (pH 6 8), 0 1 mM cystein HCl and 0 025% proteose peptone The organisms were cultured in 50 ml serum bottles filled with 20 ml medium and a nitrogen atmosphere The bottles were kept in the dark at room

0932 4739/90/0025 0369$3 50/0

temperature. Transformation to amoeboid stages was induced by adding 1.5% oxygen to the headspace of the culture bottles. Growth of the cells was followed with an inverted microscope.

Both flagellate and amoeba stages were studied in detail using bright field and differential interference contrast microscopy. Living cells as well as cells fixed with 1% glutaraldehyde were examined. The methanogenic bacteria were identified by Leitz epifluorescence microscopy [7]. Examination and photography were facilitated by use of the anti-fading compound citifluor (Agar Scientific Ltd., Stansted, Essex, GB). Nuclei were stained with the fluorescent dye 4,6-diamidino-2-phenyl-indole (DAPI). For scanning electron microscopy (SEM), flagellate cells were fixed according to Parducz [19] and transferred to coverslips coated with poly-L-lysine. The cells were dehydrated through ethanol and critical point dried. The specimens were coated with gold and examined with a JEOL scanning electron microscope (JSM-U3). For transmission electron microscopy (TEM), flagellates and amoebae were fixed in a cold mixture of 2% glutaraldehyde and 1% OsO_4 in 0.1 M sodium cacodylate (pH 7.2) for 30 min. After postfixation in 2% OsO_4 the cells were stained with 1% uranyl acetate. Dehydration was accomplished by passing the cells through a series of ethanols, followed by embedding in Epon resin. Sections were stained with lead citrate and subsequently examined with a Philips 201 or JEOL 1200 EX electron microscope.

Observations

General accounts of the organism

Two forms of the life cycle were encountered: a flagellate and an amoeba. Cysts were not found. Both forms reproduced asexually by mitotic division. The organisms were feeding on bacteria growing in the medium. For optimal growth a low redox potential was required, brought about by reducing agents and by bacterial activity. A cell density of 1.2×10^4 cells could be achieved [3]. Under normal culture conditions, the cells of the flagellate stage have a rounded anterior and a pointed posterior end, measuring about 15 × 25 µm (Fig. 1). After exposure to air the flagellate cells rapidly lose their characteristic shape and eventually die. The cells swim straight forward, without skipping, while rotating around the longitudinal axis. They show a fourfold rotational symmetry, with four nuclei, four grooves and four sets of four flagella. The flagella are of equal length, about 1.5 times the length of the cell body. The flagellar insertion is at the apex of each groove, which extends about 2/3 of the length of the cell and is located on the left hand side of the anteriorly located nucleus. The right lip of the groove follows the central axis of the cell, whereas the left lip makes a gentle concave curve. The features are represented diagrammatically in Figs. 1 and 2 and can also be seen in Figs. 6, 7 and 8.

Each nucleus contains a central nucleolus. About 85% of the cells possess 4 nuclei. Other numbers, varying between 1 and 8 nuclei, were encountered. In growing cultures large, slowly swimming cells with more than four bundles of flagella were observed. When treated with DAPI they were found to contain 8 nuclei. These cells gradually elongated and divided into two daughter cells, each of which divided subsequently again. The whole process of twofold cytokinesis lasted about 20 minutes.

In the centre of the cell a round, light-refractive body is located, measuring about 7 µm in diameter (Fig. 3). Squashing of the cells revealed the presence of many rodlike bacteria within this globule. When the preparation was studied by means of epifluorescence microscopy, the bacteria showed a bluish autofluorescence, characteristic for methanogenic bacteria. In unsquashed preparations the whole globule was found to exhibit fluorescence due to the tight aggregation of the light-emitting rods (Fig. 4). The presence of a contractile vacuole could not be ascertained because of food vacuoles and light-refractive granules, which occupy the anterior region of the cell.

The amoeboid cells could only be obtained by adding 1.5% of oxygen to the headspace of culture bottles [3].

Fig. 1. Diagrammatic representation of the *Psalteriomonas lanterna* flagellate stage, illustrating the general organization of the cell. G = globule; N = nucleus. For details see text.

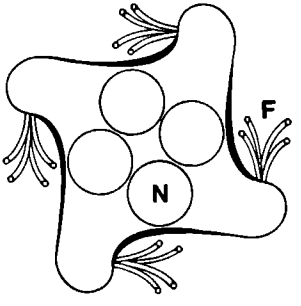
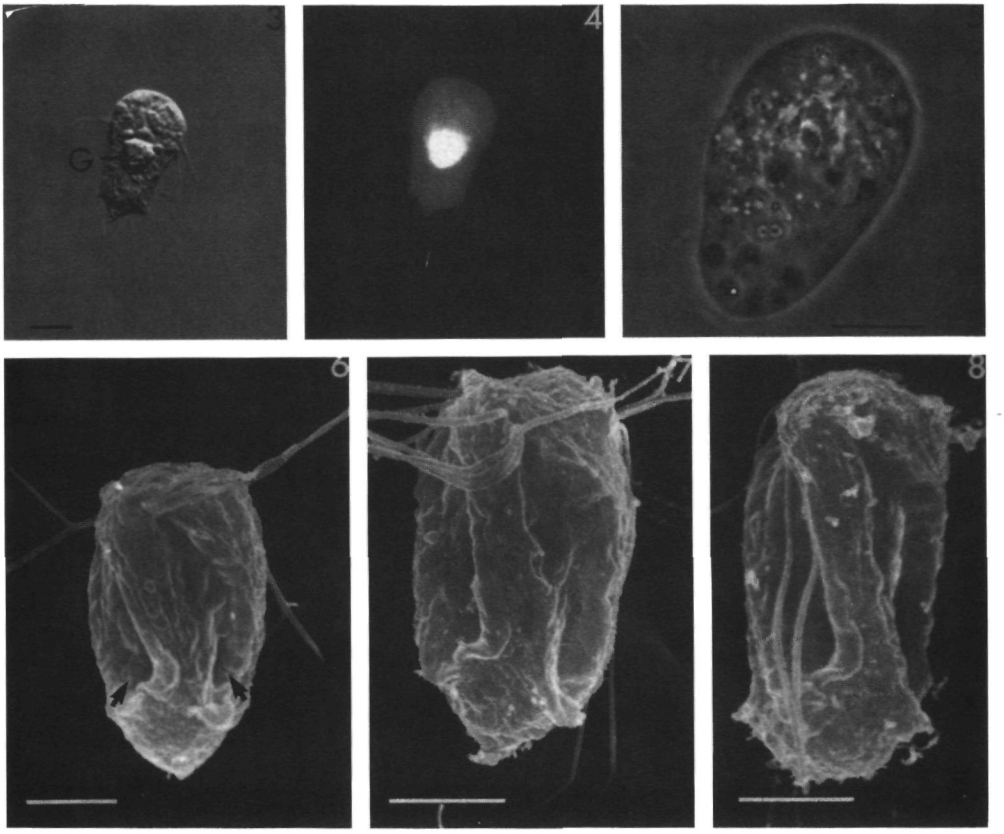
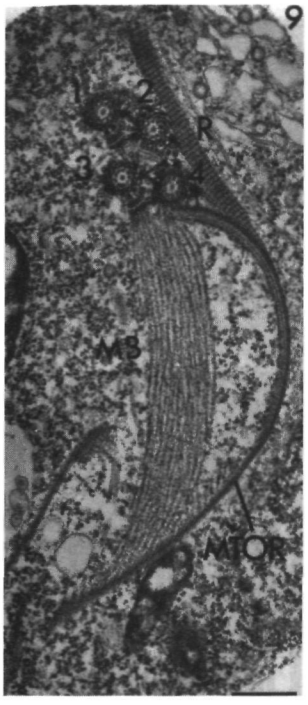


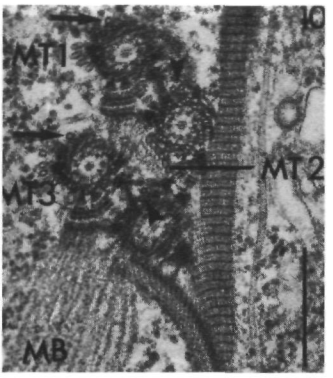
Fig. 2. Schematic transverse section through the anterior part of the flagellate. In reality, the four nuclei (N) are localized below the insertion of the flagella (F).

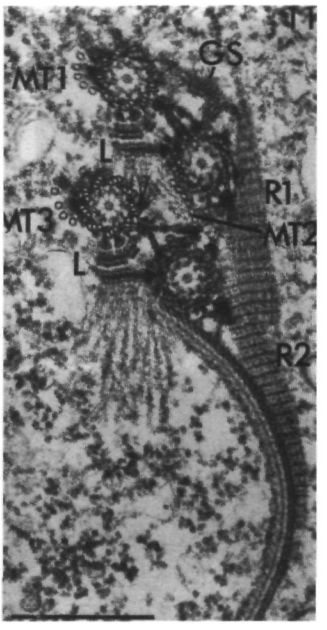


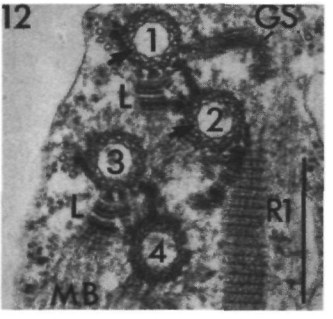
Figs. 3–5. Light microscopy, bar = 10 μm. – Fig. 3. Flagellate cell (differential interference contrast) showing the general morphology and the laterally inserted flagella. A large globule (G) is located centrally. – Fig. 4. Same cell using epifluorescence microscopy. The globule shows the typical fluorescence of methanogenic bacteria. – Fig. 5. Amoeba cell with advancing pseudopodium. – Figs. 6–8. Scanning electron micrographs of the flagellate cell. – Figs. 6, 7. Two of the four grooves are seen (arrows). A set of four flagella arises from the apex of each groove. In Fig. 8 two flagella are extended along a groove. Bar = 5 μm.

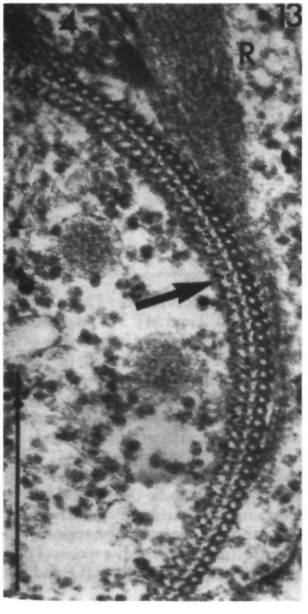
Figs. 9–15. Transverse sections through the kinetosomes which are numbered 1–4. Bar = 0.5 μm. Abbreviations: FM = fibrous material, GS = gully-like structure, L = lamella, LF = left fibre, MB = microfibrillar bundle, MT 1, MT 2 and MT 3 = microtubular rows 1, 2 and 3, MTOR = microtubule-organizing ribbon, N = nucleus, R = rhizoplast, RF = right fibre. – Fig. 9. General view of the two sets of kinetosomes 1–2 and 3–4 with their associated main elements. A rhizoplast (R) connects the kinetosomes with the curved ribbon (MTOR). Like the strings of a harp, a microfibrillar bundle (MB) passes from the kinetosomes to the posterior region of the MTOR. – Fig. 10. Detail of 9, showing the three microtubular rows (MT 1, MT 2 and MT 3). Pairs of microtubules (arrow heads) and single ones (arrow) are found at corresponding locations. – Fig. 11. Section through the basal level of the kinetosomes, showing the cartwheel structure. Two rhizoplasts, R 1 and R 2, are attached to the kinetosomes 2 and 4, respectively. Interkinetosomal strands and lamellae (L) can be seen. The microtubular row (MT 2) near kinetosome 2 displays a similar organization as the MTOR. – Fig. 12. Section through a more distal level of the kinetosomes. An internal thread connects the microtubular doublets of the kinetosomes (arrow). A gully-like structure (GS) emerges from kinetosomes 1 and 2. The microfibrillar bundle (MB) extends from lamellae near kinetosomes 3 and 4. – Fig. 13. Detail of the MTOR. The curved ribbon of microtubules is coated with paracrystalline material on the concave side (arrow). A rhizoplast (R) is attached to the ribbon. – Fig. 14. Section through the transition region of the kinetosomes. Fibrous material (FM) connects the MTOR with the nucleus (N). The two anterior kinetosomes show the terminal plate and fibres radiating outward. These structures are better seen in Fig. 15. Here the MTOR is split into two separate fibres (LF and RF). The gully-like structure (GS) is accompanied by microtubules. ▶

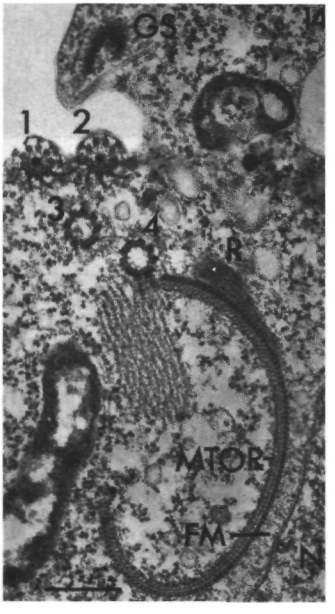

9
R
MB
MTOR

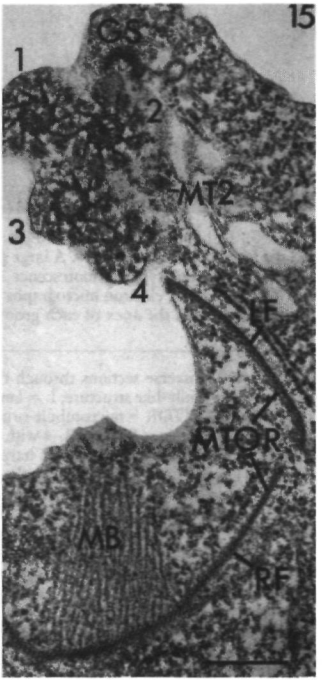

10
MT1
MT2
MT3
MB

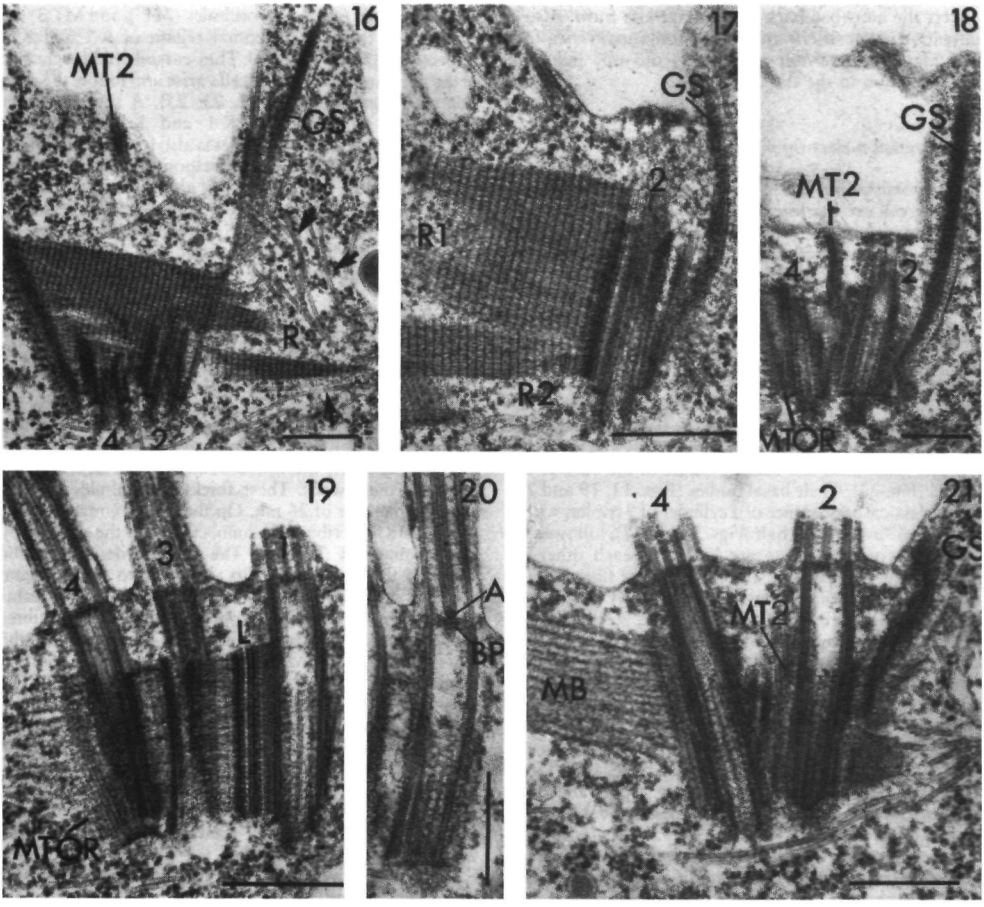

11
GS
MT1
L
R1
MT3
MT2
L
R2


12
GS
1
L
2
3
L
R1
4
MB


13
R


14
GS
1
2
3
4
R
MTOR
FM
N


15
GS
1
2
MT2
3
4
RF
MTOR
MB
RF



Figs. 16–21. Longitudinal sections through the cell and the kinetosomes. Bar = 0.5 µm. Abbreviations: A = axosome, BP = basal plate, GS = gully-like structure, L = lamella, MB = microfibrillar bundle, MT = microtubular row, MTOR = microtubule-organizing ribbon, R = rhizoplast. – Fig. 16. Section showing the origin of a rhizoplast (R) and spaced microtubules (arrows). The gully-like structure (GS) and the microtubular row (MT 2) are seen. – Fig. 17. Two rhizoplasts (R 1 and R 2) are linked to kinetosome 2. In its proximity the origin of the gully-like structure (GS) is located. – Fig. 18. The MTOR is linked to the base of kinetosome 4 and the microtubular row MT 2 is attached to kinetosome 2, as well as the gully-like structure. – Fig. 19. Section through the kinetosomes 1, 2 and 3, showing the cartwheel structure and periodic structures of the interkinetosomal lamellae (L) between them. – Fig. 20. A single kinetosome, showing the axosome (A) from which the central pair of microtubules arises. The basal plate separates the distal part of the kinetosome from the axoneme. – Fig. 21. Section through kinetosomes 2 and 4. The microfibrillar bundle (MB) is linked to kinetosome 4. The origin of the gully-like structure (GS) as well as the microtubular row MT 2, is localized near kinetosome 2. The MTOR originates from a lamella near kinetosome 4.

This concentration did not harm the organisms. Only a small minority of the flagellates could be transformed to the amoeba stage. All attempts were unsuccessful to induce a flagellate-to-amoeba transformation by changes in pH, temperature, salt concentrations or the composition of the medium. The amoeba cells in the oxygenated culture bottles could be isolated and cultivated in pure culture. The cells were found to be mononucleated and to have a *limax*-type of organization (Fig. 5). No fluorescent bacteria could be encountered. Attempts were unsuccessful to

convert the amoebae back to the flagellate form. Also a cultivation under strictly anaerobic conditions in coculture with *Methanobacterium formicicum* did not induce a transformation to the flagellate stage.

Transmission electron microscopy

Mastigont system The most prominent structures in the flagellate cell are the four mastigont systems. Each mastigont system is quite complex, consisting of four kinetosomes with associated structures. The kinetosomes or basal bodies are arranged in two pairs: one pair is located anteriorly, its kinetosomes are numbered K 1 (right anterior) and K 2 (left anterior). The second pair is located posteriorly, these kinetosomes are numbered K 3 (right posterior) and K 4 (left posterior). The flagella are inserted slightly subapically to the right edge of a groove (Fig. 9). They exhibit the typical 9 + 2 arrangement of microtubules. The flagella are surrounded by a flagellar membrane which is continuous with the plasma membrane (Figs. 19, 20). The membrane has fin-like bulges at the base of the flagella (Fig 23). Their basal bodies (Figs. 11, 19 and 20) have a classical appearance of a cylinder of 9 triplets with a "cartwheel" in the basal half (Figs. 10 and 11), followed by a zone where the triplets are linked to each other by internal thin threads (Fig. 12). The central fibres of the axoneme arise from a basal plate, which marks the transitional zone (Fig. 20). At this point, electron-dense anchoring fibres radiate outwards, to attach on the flagellar membrane (Fig. 15). The basal bodies are interconnected by osmiophilic strands and lamellae, which are found on corresponding places in each pair. These interkinetosome connectors are composed of microfibrillar material and exhibit a periodicity in transverse (Figs. 10–12) and in longitudinal sections (Fig. 19). Links between the two kinetosomes forming a pair and links between the two pairs of kinetosomes could be distinguished (Figs. 10, 11).

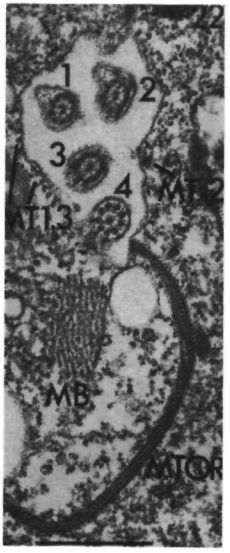
Associated with the kinetosomes are structures which form the cytoskeleton and determine the cell shape. Anteriorly, there is an electron-dense, gully-like structure (GS) associated with K 1 and K 2 which is shown in transverse sections (Figs. 11, 14, 15) and in longitudinal sections (Figs. 16–18, 21). It functions probably as a strut in the apex of the flagellar aperture.

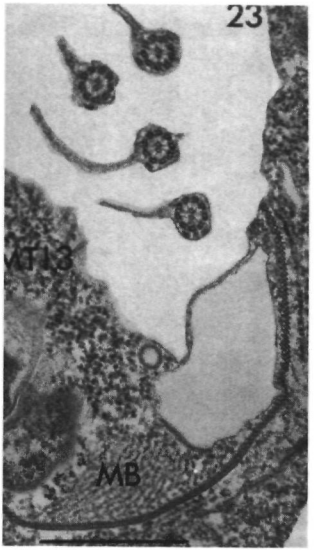
Two rows of 6 to 8 microtubules (MT 1 and MT 3) are attached to the right proximal region of K 1 and K 3, respectively (Figs. 9–12, 14). They extend on the right side of the aperture where the flagella arise and follow the right side of the groove (Figs. 22, 23, 25). A supplementary microtubule is seen near K 1 and K 3, respectively (Fig. 10). A pair of microtubules is also found anteriorly to K 2, and a second pair at a corresponding place near K 4 (Fig. 10) Linked to K 2, a row of about 6 microtubules is found which displays at its proximal level the same double layer structure as the MTOR (described below) (Figs. 10, 11). This row courses on the left side of the flagellar aperture and continues on the left side of the groove (Figs. 22, 25).

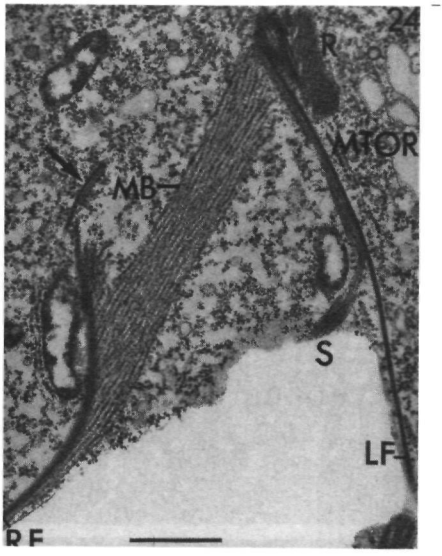
The most prominent structure in the mastigont system is a single row of microtubules, which is linked to the base of K 4 (Figs 9–11). In cross-sections this ribbon appears to be curved. Its concave side is coated with a double layer of paracrystalline material (Fig. 13). The ribbon acts as the source of new microtubules and is called the "microtubule-organizing ribbon" (MTOR). In comparison to cytoplasmic microtubules, the microtubules of the MTOR have relatively thick walls These thick microtubules have an average diameter of 26 nm. On the lower, convex side of the MTOR, the ribbon is connected with the nucleus by fibrous material (Fig. 14). The MTOR determines the shape of the groove. It splits distally into two separate fibres of microtubules (Fig. 15). In this region the paracrystalline double layer disappears. The separated fibres support the edges of the groove. One fibre arises from the anterior part of the MTOR and runs under the plasmamembrane of the left edge of the groove (Figs. 24–27). The second fibre curves across the nucleus and descends along the right lip of the ventral groove (Figs. 24–27). From the paracrystalline double layer on the anterior side of the MTOR, a microfibrillar string arises (Fig. 24). This string is first attached to the left fibre but then crosses to join to the right fibre (Figs 24–27). It is the nucleating centre for spaced microtubules which reinforce the bottom of the ventral groove (Figs. 25, 27).

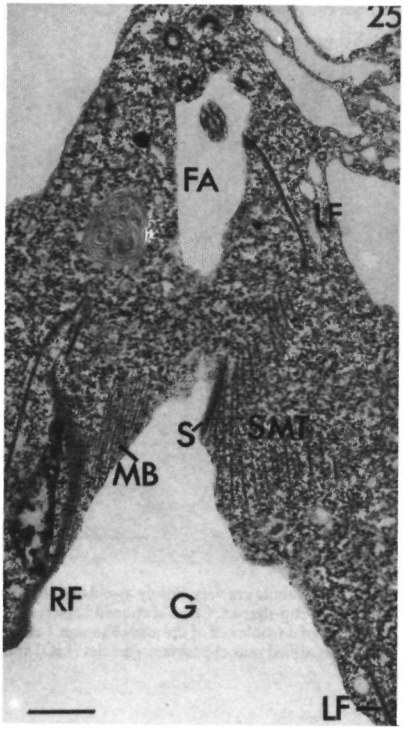
With the groove another prominent structure is associated. It is a bundle of microfilaments (MB) which arises from the lamellae posterior from K 3 and, at a more distal level, also from K 4 (Figs. 9, 10, 14, 21). This bundle connects the lamellae with the groove side of the right fibre (Figs. 9, 21, 24).

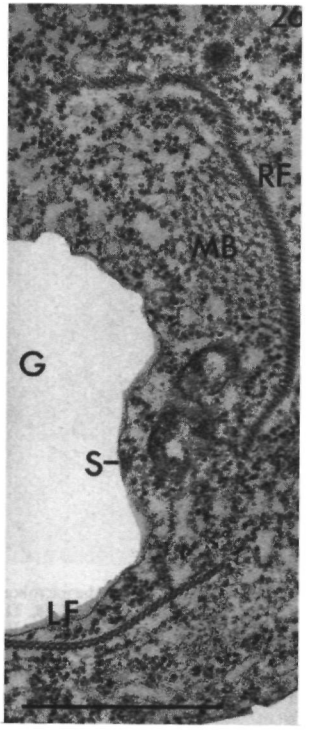
Figs 22–27. Sections through the flagellar aperture and the cell groove. Bar = 1 µm Abbreviations F = flagellum, FA = flagellar aperture, G = groove, LF = left fibre, MB = microfibrillar bundle, MT 1, MT 2 and MT 3 = microtubular rows, MTOR = microtubule organizing ribbon, R = rhizoplast, RF = right fibre, S = microfibrillar string, SMT = spaced microtubules – Fig 22. Transverse section at the base of the flagellar aperture Three microtubular rows (MT 1, MT 2 and MT 3) reinforce the wall of the funnel-like flagellar aperture – Fig 23. The flagellar sheaths have fin-like projections – Fig 24. Longitudinal sections through the microfibrillar bundle (MB) and the microtubules of the MTOR, which is separated in a left fibre (LF) and a right fibre (RF) to give shape to the groove (G). A microfibrillar string (S) extends from the left side of the MTOR A part of the distal right side of the MTOR is visible. – Fig. 25. Oblique section through the anterior part of the cell where the flagella (FA) arise. The groove (G), microtubular fibres (LF and RF), the string (S) and spaced microtubules (SMT) are shown. – Fig 26 Transverse section through the groove (G), showing the left (LF) and right fibre (RF) To the latter fibre the microfibrillar bundle (MB) is attached The microfibrillar string (S) is found near the membrane – Fig. 27. More posteriorly in the groove, the two supporting fibres (LF and RF) are more separated Here the string (S) is proximal to the right fibre (RF) Spaced microtubules (arrows) associated to dense material, occupy the bottom of the groove (G).

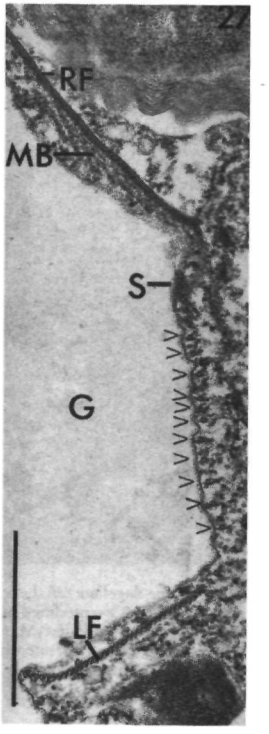

22
1
2
3
4
MB
MTOR


23
MB


24
R
MTOR
MB
S
LF


25
FA
LF
S
SMT
MB
RF
G
LF


RF
MB
G
S
LF


RF
MB
S
G
LF

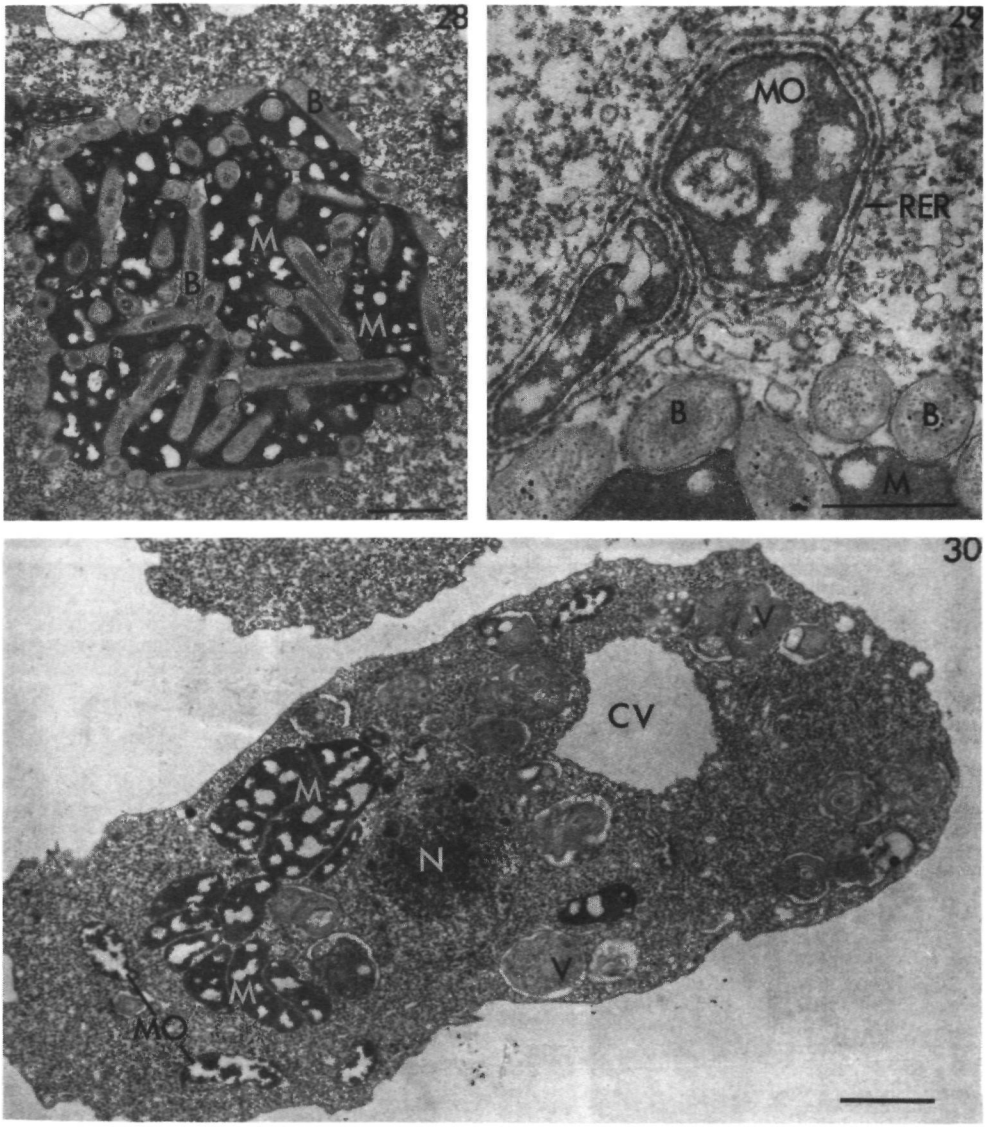


Fig. 28. In the flagellate cell the endosymbiotic bacteria (B) and the microbodies (M) of the globule are very closely associated. Bar = 1 μm. – Fig. 29. Two modified mitochondria (MO) surrounded by RER. The upper one is cup-shaped. Cross-sectioned bacteria (B) and parts of the microbodies (M) are visible. Bar = 0.5 μm. – Fig. 30. Longitudinal section of a whole cell of the amoeba stage. Left to the nucleus (N) a cluster of microbodies (M) is localized, furthermore food vacuoles (V), modified mitochondria organelles (MO) and presumably a contractile vacuole (CV) are present. Bar = 1 μm.

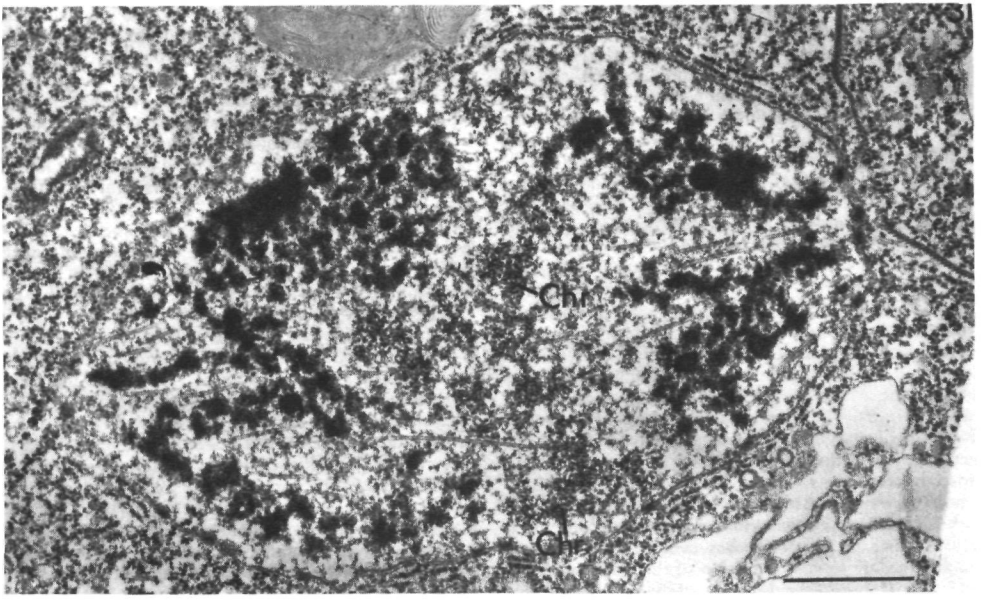


Fig. 31. Flagellate nucleus in anaphase-telophase stage. Tracts of microtubules in the nucleoplasm are evident. Nucleolar material is slightly scattered and has been separated in two polar masses. Less dense material (chromosomes) is found in the area between the polar masses (Chr). Bar = 1 μm.

One can at least distinguish two periodic roots (Figs. 9–11, 16), being linked to each other. They originate from electron-dense material anterior to the insertion of the kinetosomes, just below the cell surface (Figs. 9, 16). From this point of attachment, microtubules also fan out. Each periodic root is linked to a pair of kinetosomes, viz. R 1 to K 1 and K 2, and R 2 to K 3 and K 4. Obviously, R 2 arises more posteriorly than R 1 (Fig. 11). The periodic roots are linked to the proximal region of the MTOR (Figs. 9, 10, 12, 13). The striated rootlets have alternating dark and light bands. Within the light band a less marked dark band is found. The periodicity of the dark and light bands is about 19 nm.

Other organelles. Spread through the cytoplasm elongated to cup-shaped organelles are found, which are surrounded by rough endoplasmic reticulum (Fig. 29). They contain clear zones and electron-dense, granular material. Cristae are not found. These organelles are believed to be modified mitochondria (see Discussion). A Golgi apparatus is absent. The food vacuoles are mainly located in the anterior part of the cell. They contain bacteria in various stages of digestion as well as myelin-like figures (Fig. 30).

The methanogenic endosymbionts are exclusively found in the globular structure in the center of the flagellate cell. They are closely associated with microbody-like organelles. This bacteria-microbody complex (BMC) is not enclosed within a membrane (Figs. 28, 29). The slender, rod-like bacteria have conical pointed ends. Each bacterium is separated from the cytoplasm or microbodies by a membrane (Fig. 29). The endosymbiont resembles *Methanobacterium formicicum*, a methanogen found in several sapropelic protozoa [10, 24]. The microbodies of the BMC are highly osmiophilic (Fig. 28). In contrast to the modified mitochondria they are never associated with RER. Also in flagellates, which had lost the endosymbionts, the microbodies are aggregated.

In the amoeboid cells, which do not harbour endosymbionts, the microbodies are associated with the nucleus (Fig. 30). The modified mitochondria are spread throughout the cytoplasm. Several vacuoles containing digested material are seen as well as a presumptive contractile vacuole.

The nuclear division pattern is promitotic. During mitosis the nuclear envelope remains intact. In the elongated nucleus an intranuclear spindle is formed. Condensed chromosomes are not seen. The nucleolus persists during the mitosis and divides into two polar lobes. At the "poles" of the nucleus no marked structures are found (Fig. 31).

Discussion

Due to the capacity to transform to a *limax*-type amoeba and due to the promitotic division pattern and the similarity of the flagellar system with that of *Tetramitus*, the organism described in this paper is obviously a member of the Heterolobosea, a class of amoeboflagellates. Genera with quadriflagellate stages are known in this class and the ultrastructural organization of the organism described in this study should be compared with that of *Naegleria* [6, 9, 12, 20], *Tetramitus* [1, 17], and *Percolomonas* [8]. The latter organism is placed *incertae sedis* in the class Heterolobosea [8]. Because of the structural differences with all members of the Heterolobosea, the organism described here is arranged in a new genus *Psalteriomonas*, with the type species *P. lanterna*. The genus name is derived from *Psalterium*, which means harp, referring to the novel harp-like structure found in transverse sections through the MTOR and the connecting microfibrillar bundle (Fig. 9). The species name is chosen because the shape of the flagellate cell and the fluorescence of the methanogenic endosymbionts during epifluorescence microscopy resemble a Chinese lantern. Under the culture conditions employed, this typical shape of the flagellate with a fourfold rotational symmetry remained constant. It is likely that the organism has evolved from a single quadriflagellate by multiplication of the karyomastigont system. That mimics the diplomonads which have evolved from an enteromonad-like flagellate with one set of flagella [5]. The Diplomonadida are characterized by a twofold rotational symmetry [5, 14], but the differences in ultrastructural organization precludes assignation of *Psalteriomonas* to this order.

In *P. lanterna* each mastigont consists of two identical pairs of kinetosomes with an own set of anchoring structures for each pair. The same elements are found on corresponding places within each pair. Just as suggested for *Tetramitus rostratus* [1] the mastigont of *P. lanterna* may have arisen as a duplication of a basic *Naegleria* type flagellar apparatus. In the biflagellate *N. gruberi* the mastigont consists of two kinetosomes, a periodic root, interkinetosomal lamellae (or links) and two "spurs" which resemble the MTOR of *P. lanterna*. In *P. lanterna* the same structures are found in duplicate. Such a double pair of kinetosomes was also noticed in *Tetramitus rostratus* [1]. However, in this organism the anterior pair has a different orientation.

The kinetosomes of *P. lanterna* are connected by interkinetosomal lamellae and strands. These links also make connections between the kinetosomes and the anchoring structures. Interkinetosomal links, though less complicated, are also present in the mastigont system of *T. rostratus*. Less clear is the organization of lamellae and strands in *Percolomonas cosmopolitus*. However, the poor preservation of the organism hampers a detailed study in this respect.

P. lanterna has two well-developed striated roots. The periodicity of the roots does not significantly differ from *T. rostratus* and *Naegleria* [6]. A diagonal periodicity as in *T. rostratus* [1, 6] is not found. *T. rostratus* has one main striated root associated with the kinetosomes. There are several smaller striated roots with different lengths and orientations [1, 17]. *P. cosmopolitus* has no striated roots.

The gully-like structure and the rows of microtubules which reinforce the flagellar aperture in *P. lanterna* are obviously absent in the other amoeboflagellates. The pairs of microtubules which are associated with the left kinetosomes can also be seen in electron micrographs of *T. rostratus* in [1].

Each kinetosome in *Naegleria* is associated with a MTOR or spur. In *P. lanterna* in each couple of kinetosomes a MTOR is linked to the left and a row of microtubules to the right kinetosome, respectively. The latter row may be a remnant of a MTOR-like spur. In *T. rostratus* each pair has one MTOR and the rows of microtubules are absent. *P. cosmopolitus* has three microtubular roots. One of them displays a MTOR-like structure and is connected with the posterior left kinetosome [8], just as the main MTOR in *P. lanterna*. The structural organization of the MTOR resembles the preaxostyle or primary row found in oxymonads. Here it gives rise to microtubules of the paracrystalline axostyle [4, 16]. The heterolobose flagellates have no axostyle. Their MTOR(s) are mainly involved in the organization of the groove.

There are fundamental differences in the organization of the groove between *P. lanterna* and both other quadriflagellates. In *T. rostratus* and *P. cosmopolitus* the margins of the groove are supported by microtubular ribbons which originate from separated rootlets. In *P. lanterna* the supporting ribbons originate from a single rootlet (MTOR). A microfibrillar bundle (MB) is attached to the ribbon on the right side of the groove. This bundle is not encountered in other heterolobose flagellates investigated so far. The string, which gives rise to microtubules occupying the bottom of the groove, is absent in *P. cosmopolitus*. However, as mentioned earlier, in this case poor fixation may have prevented clear inspection of the groove. A comparison with *Tetramitus rostratus* in this respect is not possible since the organization of the groove of the latter is not clarified. A thorough ultrastructural study of the genus *Tetramitus* is now being undertaken.

The organelles surrounded by RER are possibly modified mitochondria. The members of the Heterolobosea described so far are aerobic organisms characterized by a close association of RER with mitochondria [8]. The RER surrounded organelles of *P. lanterna* could be stained with mitochondria-specific dyes [2, 3]. However, they lack internal membrane structures. This could be a fixation artifact, but could also be explained by the anaerobic mode of life of this organism. It is known from yeast grown anaerobically that the mitochondria lose their typical fine structure [15].

To our knowledge the globular microbody complex is a structure not described before. Many anaerobic sapropelic protozoa investigated so far [23] show a close association of microbody-like organelles and methanogenic endosymbionts but never to such an extent observed in *P. lanterna*. The microbody-like organelles differ from the modified mitochondria with respect to the association with RER

and the uptake of mitochondria-specific dyes [2, 3]. Until now, attempts to isolate and characterize the endosymbiotic methanogens failed, thus nothing is known about the substrate specificity of the bacteria. Possibly hydrogen is utilized which may be provided by the microbodies, indicating a hydrogenosome-like function. Such a function was recently demonstrated for the sapropelic ciliates *Plagiopyla nasuta* and *Trimyema compressum*, where hydrogenase activity could be shown in methanogen-associated microbodies [25]. A localization of hydrogenase activity within the microbodies of *P. lanterna* is under investigation now. The benefit of the association for host and endosymbiont has been extensively discussed elsewhere [22].

The nuclear division pattern of *Psalteriomonas* shows the characteristics of a closed mitosis. The information on the process is not complete, but shows the same features as that of *Naegleria* [21] and *Tetramitus* [1]. The nuclear envelope remains intact throughout mitosis, the nucleolus does not disappear, and an intranuclear spindle is formed. Centrioles are not found. The pattern of cell division with the twofold cytokinesis during the flagellate stage distinguishes *P lanterna* from the other amoeboflagellates.

The transformation of *Psalteriomonas* from flagellate to amoeba could only be induced so far by the addition of oxygen to the headspace of the culture bottles. Also in the transformation of *Tetramitus rostratus* flagellates, oxygen plays an important role. Hollande [11] discovered that a higher oxygen tension in the medium promoted the amoeboid phase. So far, there is no ultrastructural information available about the transformation process of *Psalteriomonas*. The amoeba cells are devoid of all the components of the mastigont system. A transformation back to the flagellate stage was not observed.

Diagnosis

Genus Psalteriomonas. Free-living heterolobosean amoeboflagellates from freshwater live under low oxygen pressure or anaerobically. No cyst stage known. The cells lack a Golgi apparatus, mitochondria have no clearly defined cristae. The nuclear division pattern shows a closed mitosis. Amoebae have a *limax* shape. The flagellate cell bears four flagella of equal length at the apex of the cell groove. A complex microtubular ribbon is attached to the kinetosomes. Two microtubular fibres arise from the ribbon, supporting the margins of the groove. A bundle of microfilaments is attached to the groove side of the right fibre.

Psalteriomonas lanterna n. sp. With characters of the genus. The flagellate stage is quadrinucleated with four cell grooves and a fourfold rotational symmetry. The grooves extend along two-thirds of the body length. Multiplication is by a twofold cytokinesis. Endosymbiotic methanogenic bacteria are closely associated with hydrogenosome-like microbodies to form a globular structure in the centre of the cell. The amoeba stage is mononucleated and bacteria-free.

Acknowledgement

We wish to thank Miss J. van Westreenen for making the drawings of the organism.

References

1 Balamuth W., Bradbury P. C. and Schuster F. L. (1983) Ultrastructure of the amoeboflagellate *Tetramitus rostratus* J. Protozool., *30*, 445–455
2 Broers C. A. M., Goosen N. K., Stumm C. K. and Vogels G. D. (1989): Endosymbiosis of methanogenic bacteria in a sapropelic flagellate. J. Protozool., *36*, 10A (suppl.) *55*.
3 Broers C. A. M., Stumm C. K. and Vogels G. D. (1988). A heterolobose amoeboflagellate associated with methanogenic bacteria In Lloyd D., Coombs G. H. and Paget T. A. (eds.): Biochemistry and physiology of anaerobic protozoa, pp. 22–31. Harwood Publishers, London.
4 Brugerolle G. (1970) Sur l'ultrastructure et la position systematique de *Pyrsonympha vertens (Zooflagellata Pyrsonymphina)* C. R. Acad. Sc. Paris, *270*, 966–969.
5 Brugerolle G. (1975). Etude ultrastructural du genre *Lnteromonas* da Fonseca (Zoomastigophorea) et révision de l'ordre des Diplomonadida Wenyon. J. Protozool., *22*, 468–475
6 Dingle A. D. and Larson D. E. (1981) Structure and protein composition of the striated flagellar rootlets of some protists BioSystems, *14*, 345–358.
7 Doddema H. J. and Vogels G. D. (1978): Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol., *36*, 752–754.
8 Fenchel T. and Patterson D. J. (1986). *Percolomonas cosmopolitus* (Ruinen) n. gen., a new type of filter feeding flagellate from marine plankton. J. Mar. biol. Ass. U.K., *66*, 465–482.
9 Gardiner P. R., Miller R. H. and Marsh M. C. P. (1981). Studies on the rhizoplast from *Naegleria gruberi* J. Cell Sci., *47*, 277–293.
10 Goosen N. K., Horemans A. M. C., Hillebrand S. J. W., Stumm C. K. and Vogels G. D. (1988). Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont *Methanobacterium formicicum* Arch. Microbiol., *150*, 165–170.
11 Hollande A. (1942): Etude cytologique et biologique de quelque Flagelles libres. Arch. Zool. expér. et gén., *83*, 1–268.
12 Larson D. E. and Dingle A. D. (1981) Isolation, ultrastructure, and protein composition of the flagellar rootlet of *Naegleria gruberi*. J. Cell Biol., *89*, 424–432.
13 Lauterborn R. (1901): Die "sapropelische" Lebewelt. Zool. Anz., *24*, 50–55.
14 Lee J. J. (1985) Diplomonadida. In Lee J. J., Hutner S. H. and Bovee E. C. (eds.). Illustrated guide to the protozoa, pp. 130–134. Society of Protozoologists, Allen Press, Lawrence, Kansas.
15 Lloyd D. (1974): The mitochondria of microorganisms. Academic Press, London.
16 McIntosh J. R., Ogata E. S. and Landis S. C. (1973): The axostyle of *Saccinobaculus* I. Structure of the organism and its microtubule bundle J. Cell Biol., *56*, 304–323.
17 Outka D. F. and Kluss B. C. (1967): The amoeba-to-flagellate transformation in *Tetramitus rostratus* II. Microtubular morphogenesis. J. Cell Biol., *35*, 323–346.
18 Page F. C. and Blanton R. L. (1985): The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica, *21*, 121–132.

19 Parducz B. (1967): Ciliary movement and coordination in ciliates. Int. Rev Cytol., *21*, 91–128.
20 Patterson M., Woodworth T W., Marciano-Cabral F. and Bradley S. G. (1981): Ultrastructure of *Naegleria fowleri* enflagellation. J. Bacteriol., *147*, 217–226.
21 Schuster F. L. (1975). Ultrastructure of mitosis in the amoeboflagellate *Naegleria gruberi* Tissue and Cell, *7*, 1–12.
22 Stumm C. K. and Zwart K. B. (1986): Symbiosis of protozoa with hydrogen-utilizing methanogens. Microbiol. Sci., *3*, 100–105.
23 Van Bruggen J J. A , Stumm C. K. and Vogels G. D. (1983): Symbiosis of methanogenic bacteria and sapropelic protozoa Arch. Microbiol., *136*, 89–95
24 Van Bruggen J. J A , Zwart K. B., Van Assema R. M., Stumm C. K. and Vogels G. D. (1984). *Metanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch. Microbiol., *139*, 1–7.
25 Zwart K. B., Goosen N. K , van Schijndel M W , Broers C. A M., Stumm C. K and Vogels G D. (1988): Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum* J. Gen. Microbiol., *134*, 2165–2170.

Key words: Psalteriomonas lanterna gen. nov., sp. nov. – Amoeboflagellate – Endosymbiosis – Mastigont – Anaerobiosis

Cees A. M. Broers, Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands

CHAPTER 2

OXYGEN RESPONSES OF THE FREE-LIVING ANAEROBIC AMOEBOFLAGELLATE *PSALTERIOMONAS LANTERNA*

Cees A.M. Broers, Ron J.M. Berkhout, Lin Yu Hua, Claudius Stumm and Godfried D. Vogels (1992), FEMS Microbiology Ecology, in press.

1. SUMMARY

The effects of O_2 on growth of the anaerobic amoeboflagellate *Psalteriomonas lanterna* were studied. The organism tolerates low oxygen tensions (about 1% O_2 atm. sat.) and under these conditions growth was stimulated in mixed populations. Catalase could not be found in the cells, whereas superoxide dismutase was present. Addition of O_2 resulted in loss of the methanogenic endosymbionts and favoured the transformation to amoeba cells. Symbiont-free cells did not grow under anaerobic conditions probably due to the accumulation of H_2.

2. INTRODUCTION

Psalteriomonas lanterna is a schizopyrenid amoeboflagellate discovered recently [1]. The organism contains methanogenic bacteria as endosymbionts closely associated with microbodies located as a globular body in the centre of the cell [1,2]. The microbodies are hydrogenosomes since they show hydrogenase activity [3]. An interspecies hydrogen transfer is thought to be the physiological background of the association [4,5]. Probably both organisms profit from the association: the methanogenic bacteria utilize hydrogen released by the hydrogenosomes. Thus a low internal H_2 tension is maintained, which enables the protozoan cell to perform oxidative reactions that are otherwise thermo-dynamically unfavourable [4]. The association of hydrogenosomes and methanogens is not essential for anaerobic growth since Trimyema compressum was able to grow without endosymbionts [5-7]. In addition to the hydrogenosomes, other organelles were found in *P. lanterna*, which show similarities with mitochondria [1-3]. Until now, the simultaneous occurrence of hydrogenosomes and mitochondria in one organism was unknown. For this reason P. lanterna appeared to be an interesting candidate to study its response to oxygen.

In this paper we report on the effects of oxygen on *P. lanterna* and the consequences of the loss of the methanogenic endosymbionts for the organism. Some preliminary results of this study have been published elsewhere [2].

3. MATERIALS AND METHODS

P. lanterna was cultivated in a liquid medium (PM) with the following composition: 7 mM K/Na phosphate buffer pH 6.8, 0.1 ml/l (v/v) Pfennig's metal solution [8], 0.025% proteose peptone, 0.1 mM cystein HCl, and 0.5 ml/l of a 1% resazurine solution. The medium was dispensed in 20 ml quantities in 50 ml serum bottles which were closed with butyl rubber stoppers and aluminium seals. The culture bottles were evacuated for 4 minutes and gassed with oxygen-free nitrogen (130 kPa). After autoclaving (15 min, 120°C) the bottles were inoculated with 1 ml of a growing flagellate culture with a hypodermic syringe. Together with the inoculum a mixture of undefined food bacteria was transferred. The cells were grown at 24°C. For experiments on responses to different oxygen tensions, the desired amount of oxygen was injected into the headspace of the culture bottles with a hypodermic syringe.

3.1. *Cocultivation with methanogenic bacteria*

Two strains of Methanobacterium formicicum (DSM 3636 and 3637), isolated from *Metopus striatus* and *Pelomyxa palustris* [4,9], were pregrown in the medium described by Balch et al. [10] under an atmosphere of 80% H_2 and 20% CO_2 at 37°C. The cells were harvested by centrifugation (20 min, 12 000 x g) and washed twice in PM medium and resuspended in this medium. All manipulation was performed under anaerobic conditions. The methanogenic bacteria were used in cocultures with endosymbiont-free *P. lanterna*.

3.2. *Microscopy*

Methanogenic bacteria were visualized by epifluorescence microscopy [11]. Growth of the protozoan cultures was followed under an inverted microscope (Leitz, Labovert). Enumeration of the amoeboflagellates was done by means of a counting chamber with samples fixed in Lugol's solution.

For electron microscopy preparations were made as previously described [1] and examined in a Philips EM 201.

3.3. *Analytical procedures*

Methane production was measured according to the method of Hutten et al.[12].

Hydrogen in the gasphase was measured by gas chromatography according to Goosen et al. [13].

For demonstration of enzyme activities the protozoa were concentrated and separated from their food bacteria by the use of an electromigration technique [publication in preparation]. Subsequently, the cells were pelleted by centrifugation (5 min at 800 g) and washed twice in anaerobic PM medium. For the demonstration of cytochrome oxidase activity, cells pregrown anaerobically or under an atmosphere of 2% O_2 were incubated for 1 h at room temperature with p-aminodiphenylamine according to Burnstone [14], or with 3,3-diaminobenzidine tetrahydrochloride according to Seligman [15]. Catalase activity was demonstrated by the transfer of a dense cell suspension to a drop of 3% aqueous hydrogen peroxide. Superoxide dismutase (SOD) activity was determined by illumination of the amoeboflagellates in a reaction mixture containing riboflavin and nitro blue tetrazolium salt according to Goosen et al. [16].

4. RESULTS

Exposure of *P. lanterna* to atmospheric oxygen was found to be very harmful to the cells. Within 10 minutes cells prepared as wet mounts or in drops of medium exposed to air lost their characteristic lantern shape, rounded up and became immobile. However, in preliminary experiments the organism had been shown not only to survive at low concentrations of O_2, but also to grow more quickly when 2% O_2 was added to the headspace of the culture bottles [2]. To study the oxygen tolerance of *P. lanterna*, a range of O_2 concentrations (from 1 to 10% O_2) was tested. The oxygen gas was added once at the start of the culture and was not replenished during further incubation. The medium, which contained resazurine as a redox indicator, turned from pink to colorless within 2 to 5 days (depending on the O_2 concentration applied). The decoloration indicates a lowered redox potential due to bacterial growth and might indirectly indicate the disappearance of O_2. The flagellates not only survived O_2 pulses of up to 7.5%, but with increasing O_2 doses (with a maximum of 5%, Fig. 1a) even an increase of cell yield was observed. The flagellates were enumerated after 16 days, when the medium in all culture flasks had become colorless. The increase of the cell yield is probably not directly attributable to a direct effect of O_2 on the flagellates, but to an increased availability of food bacteria, as the densities of the food bacteria

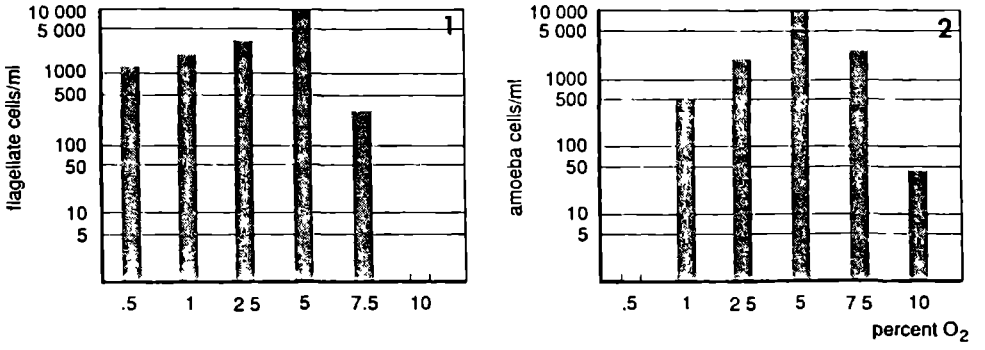


Fig. 1. Growth population density of flagellates after 16 days of cultivation under different O_2 concentrations in the initial gas phase. Cultures were started with 20 flagellate cells/ml.
Fig. 2. Culture density of amoebae after 12 days of cultivation under different O_2 concentrations in the initial gas phase. Cultures were started with 8 cells/ml.

were found to be positively correlated with the O_2 concentration of the pulses (results not shown). On exposure of the flagellates to low oxygen tensions, the swimming velocity increased and the cell shape changed: the cells became elongated and showed a protrusion of the anterior end (Fig. 3, 4).

Another consequence of the addition of oxygen to the culture bottles was the loss of methanogenic endosymbionts by the flagellates (Fig. 5). After one or two transfers to fresh medium under an atmosphere of 2% O_2, or after refreshment of the oxygenated gas phase twice a week, about 80 to 100% of the cells were free of methanogenic endosymbionts. Those cells, which still contained endosymbiotic bacteria, harboured apparently the normal quantity of about 200 methanogens. Symbiont-free flagellates were not able to grow under strictly anaerobic conditions, unless the nitrogen atmosphere of the culture bottles was refreshed twice a week.

Finally, in cultures which had obtained a pulse of 2 to 5% O_2, a transformation of the flagellates to a mononucleated amoeba stage occurred. It was not possible to determine the percentage of flagellates which underwent transformation, because both flagellates and amoebae continued to multiply. The first amoeba cells appeared a few days after the addition of O_2, indicating that the process of transformation was slow. After reaching the stationary growth phase, the flagellates died within about 2 days. The amoeba cells, however, survived for 1 to 2 more weeks. In amoeboid cells methanogenic endosymbionts

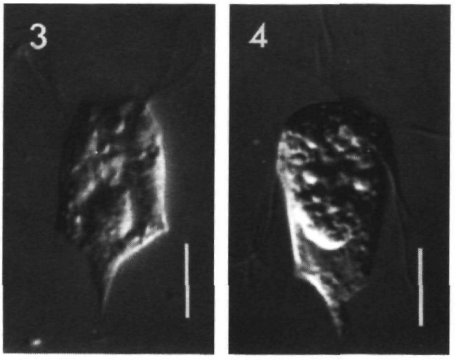


Fig. 3-4. Light micrographs (differential interference contrast) of the flagellate stage of *P.lanterna*, cultured under strictly anaerobic conditions (fig. 3) or with 1-2% O_2 in the headspace (fig. 4). Note the protrusion of the anterior part of the oxygen-treated cells. Bar = 10 μm.

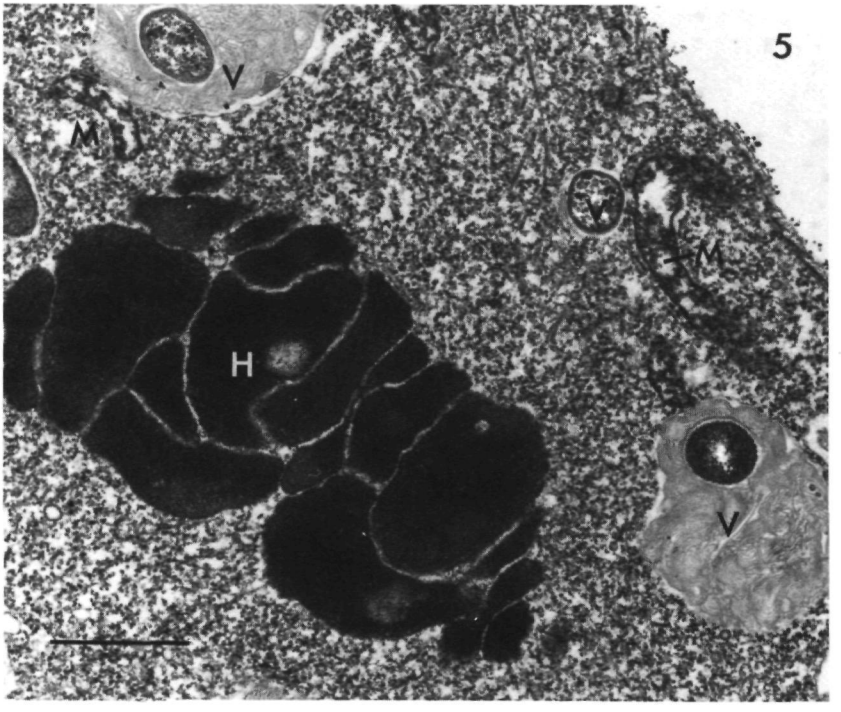
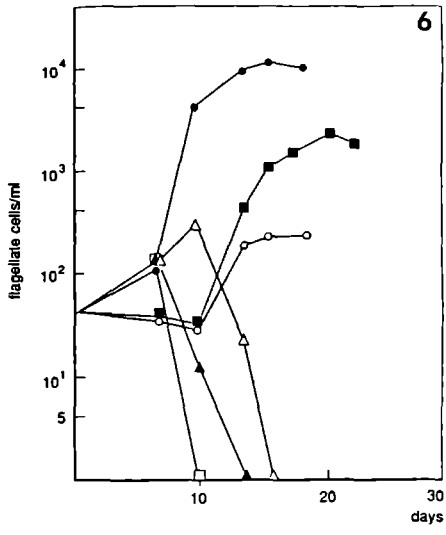


Fig. 5. Transmission electron micrograph of the posterior half of a symbiont-free flagellate cell. Aggregated hydrogenosomes (H) are still present. M = mitochondrion-like organelle; V = food vacuole. Bar = 1 μm.

were never found. Like the endosymbiont-free flagellates, the cells of the amoeba stage did not grow under anaerobic conditions, unless the gas phase was refreshed twice a week. However, they tolerated pulses of higher O_2 concentrations than the flagellates (up to 10%,



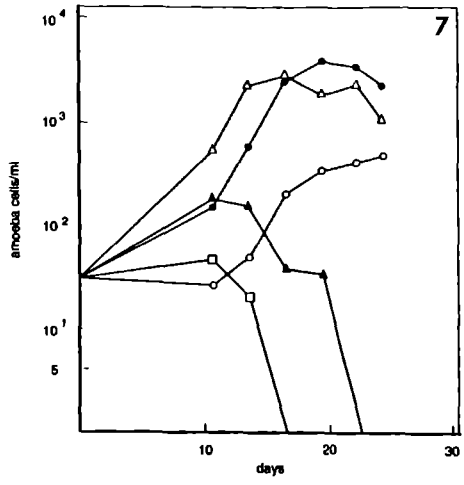


Fig. 6. Growth of *P. lanterna* flagellates under different O_2 tensions. The gas phase was changed twice a week. Symbiont-containing flagellates: (■), 100% N_2. Symbiont-free flagellates: (○), 100% N_2; (●) 1% O_2; (△), 2,5% O_2; (▲), 5% O_2; (□), 7,5% O_2.

Fig. 7. Growth of the amoebae under different O_2 tensions with an exchange of the gasphase twice a week. For symbols: see fig 7.

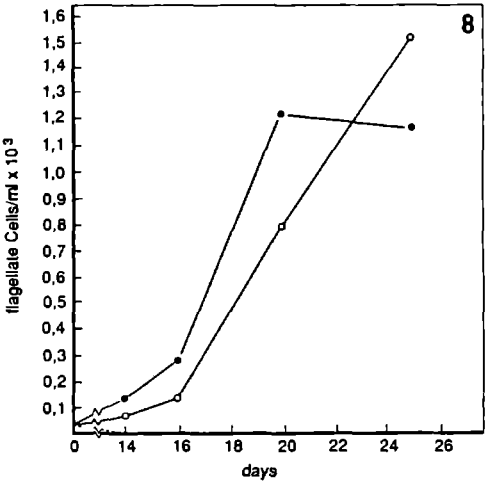


Fig. 8. Growth of symbiont-free flagellates in coculture with *M. formicicum* strain DSM 3636 (○), strain DSM 3637 (●) and without *M. formicicum* (--).

see Fig. 1b).

To study the response of both flagellates and amoebae to a more permanent oxygen atmosphere, the gas phase, containing various amounts of O_2, was exchanged twice a week. Under these conditions, 1% O_2 turned out to promote optimal growth of the flagellates (Fig. 6). The doubling time was 14 to 18 h, and after 14 to 17 days of incubation, the final cell density reached a maximum of 12 x 10^3 cells/ml. With an exchange of the nitrogen gas phase twice a week, symbiont-free cells grew slowly under anaerobic conditions and the

Table 1. Production of CH_4 and H_2 in symbiont-free flagellate cultures after 16 days of cocultivation with 2 strains of *M. formicicum*

Organism	CH_4 (μmol)	H_2 (μmol)	Flagellates (cells ml^{-1})
Flagellates	0	6	0
Flagellates +			
M. formicicum strain 3636	5.1	3	280
M. formicicum strain 3737	3	2.6	130
Food bacteria +			
M. formicicum strain 3636	4.7	2.5	0
M. formicicum strain 3637	2.3	2.6	0

final cell yield was lower than that of the symbiont-containing flagellates (Fig. 6); the doubling times were 35 and 20 h, respectively. The growth characteristics of the amoebae were similar to those of the symbiont-free flagellates under these conditions, with doubling times of 56 h under anaerobic conditions and 28 h when 1% O_2 was added to the gas phase (Fig. 7). Again it was evident that the amoebae tolerated higher O_2 tensions than the flagellates; nevertheless, a good growth was observed under an atmosphere of 2.5% O_2 (Fig. 7).

In order to study the effect of methanogenesis on the growth of *Psalteriomonas* cells under anaerobic culture conditions without refreshment of the nitrogen atmosphere, a series of experiments was undertaken.

First, symbiont-free flagellates were cocultured with two strains of Methanobacterium formicicum (5.10^7 cells/ml each), a methanogen known to consume hydrogen. After a lag period of 14 days, the flagellates started to grow, reaching densities of $1.2.10^3$ to $1.6.10^3$ cells/ml after 26 days (Fig. 8). Analysis of the headspace atmosphere revealed the presence of a considerable quantity of methane together with H_2 (Table 1). The hydrogen production might be - at least partly - attributable to the food bacteria, since hydrogen production was also found in culture bottles containing only food bacteria. The control cultures without methanogens showed no growth of the flagellates (Fig. 8). In the atmosphere of these bottles two times more H_2 was present than in co-culture with methanogens. Similar results were obtained with the amoeba stage of *P. lanterna* co-cultured with and without *M. formicicum*.

Table 2

Effect of the addition of bromoethanesulfonic acid (BrES) on growth of *P. lanterna* cells, methane excretion and H_2 production

BrES concentration	Cell density (cells ml^{-1})		CH_4 production rate (nmol h^{-1})		Inhibition CH_4 production (%)	H_2 accumulated during 4 days of incubation (μmol)
	1 day before addition	3 days after addition	Before addition	After addition		
0	600	850	10 4	12.1	0	3
0 1	710	350	15 4	1 3	91	7
1	710	350	13 3	0	100	8 5

Epifluorescence microscopy of samples from the co-cultures showed numerous *M. formicicum* cells attached to the flagellates. Methanogens were also present in the food vacuoles but were never found in the cytoplasm. Obviously the bacteria were ingested but a restoration of the endosymbiosis as found for *T. compressum* [7] did not take place, not even after a prolonged co-incubation of 3 months.

Secondly, flagellates containing methanogenic endosymbionts were treated with bromoethanesulfonic acid (BrES) in 10 mM phosphate buffer. At a concen-tration of 0.1 mM BrES methane production was strongly inhibited (Table 2). As a consequence H_2 accumulated in the culture bottles, which may explain the rapid decrease in cell density. Within 5 days the flagellates died. BrES was found to be not inhibitory to *Psalteriomonas* since no effect on cell growth was observed when concentrations up to 10 mM BrES were added to cultures with a refreshment of the headspace atmosphere.

Enzyme activities

In *P. lanterna* modified mitochondria were described earlier [1,2]. To study the presence of cytochrome oxidase activity the test of Burnstone with p-aminodiphenylamine as well as the test of Seligman with 3,3-diamino-benzidine tetrahydrochloride were performed. The tests gave negative results both with cells which were precultured under strictly anaerobic conditions and with cells cultured under an atmosphere of 2% O_2.

To test whether the observed aerotolerance could be related to catalase and superoxide-dismutase (SOD) activities the cells were subjected to cytochemical tests for the demonstration of both enzymes. When a dense suspension of symbiont-free flagellate cells was added to a 3% (v/v) H_2O_2 solution, no oxygen production was observed indicating the absence of catalase activity. For the demonstration of SOD, cells were illuminated for 45 minutes in the reaction mixture according to Goosen et al. [16]. In symbiont-free cells,

cultured under an atmosphere of 1-2% O_2, the cytoplasm, the mitochondrion-like organells and the globular body consisting of hydrogenosomes [3] were colorless, indicating the presence of SOD By contrast, in about 60% of the symbiont-free flagellates grown under strictly anaerobic conditions (with an exchange of the gas phase twice a week) the globular body was found to be SOD-negative

5. DISCUSSION

Psalteriomonas lanterna is an amoeboflagellate containing both hydrogenosomes and mitochondrion-like organelles. Although the latter organelles are not fully functional as confirmed by the absence of cytochrome c oxidase activity the organism appears to profit from low oxygen concentrations This is shown by the increased growth yield observed under these conditions. However, compared to similar sized aerobic heterotrophic flagellates, the minimum doubling times are about 4 times longer, a value characteristic for anaerobic protozoa [17, 18]. As suggested for anaerobic ciliates [19], the growth stimulation of *P lanterna* might be explained by the increased number of (aerobic) food bacteria Also a direct growth stimulation of the protozoa could have taken place by an enhanced synthesis of sterols or unsaturated fatty acids Furthermore, oxygen may replace protons as a terminal electron acceptor thus stimulating hydrogenosomal electron transport which may result in higher growth yield [20]

Superoxide dismutase might protect the amoeboflagellate against toxic superoxide radicals generated under low oxygen tensions This enzyme was found to be present in the cytoplasm as well as in the hydrogenosomes SOD was found to be present in the cytosol, the mitochondrion-like organelles and the hydrogenosomes. As shown for *Saccharomyces cerevisiae* [21] and trichomonads [22], the enzymes found in the cellular compartments of *P. lanterna* may represent different types of SOD. The hydrogenosomal enzyme might be induced by oxygen, a well-known property of some types of SOD [21,23]. The fact that catalase, another defence mechanism against toxic oxygen compounds, is absent, may account for the observation that an oxygen content of 7 5% in the culture bottles already inhibits growth of *P. lanterna* The organism may even tolerate slightly higher O_2 tensions, since resazurin might be toxic to anaerobic protozoa due to the formation of peroxide when

resazurine is oxidized.

The loss of the methanogenic endosymbionts by *P. lanterna* is probably caused by oxygen toxicity and not by a statistical outgrowth as supposed for *Trimyema compressum*. Many anaerobic protozoa are capable of consuming oxygen although this is not coupled to energy conservation [24]. In this way free-living anaerobic ciliates may maintain an anaerobic intracellular environment thus protecting the methanogens [25]. However, *P. lanterna* is much smaller than these ciliates and its size seems to be insufficient to protect the endosymbionts by diffusion limitation.

The inability of symbiont-free amoeboflagellates to grow under anaerobic conditions without refreshment of the gas phase is possibly due to accumulation of H_2. In this respect *P. lanterna* differs from other anaerobic, symbiont-free ciliates, which show no loss in growth under comparable conditions [6,26]. However, the culture conditions of *P. lanterna* might be quite different to those of the ciliates, e.g. the absence of sulfate compounds and the replacement of sulfide by glutathions as reducing agent in the medium. The culture medium is probably not favourable for sulfate reducers, which are also capable of utilizing H_2. Though it is possible that *P. lanterna* profits under laboratory conditions from the endosymbiotic relationship by the maintenance of a low intracellular $p[H_2]$, this benefit might be less significant in the natural habitat of the organism, where the external H_2 tension is low [18,25]. Furthermore, anaerobic protozoa might profit from the endosymbiosis by utilizing organic compounds excreted by the methanogens [18,26]. The observed differences in growth rate and cell yield between the anaerobically cultured symbiont-containing and symbiont-free amoeboflagellates (with a refreshment of the gas phase) as well as the symbiont-free cells, being cocultured with M. formicicum might indicate such a transfer of dissolved organics.

As reported earlier [1,2] elevated O_2 levels (2-5%) favoured the transformation to amoebae. The process of transformation is slow as compared to the related aerobic amoeboflagellates Naegleria and Tetramitus. Also for *Tetramitus rostratus* O_2 was found to play an important role in the process of transformation [27,28].

Many facets of the organism concerning the response to oxygen deserve further attention. Further studies with axenic cultures should enable us to elucidate some of the remaining problems regarding the organism.

REFERENCES

1. Broers, C.A.M., Brugerolle, G., Stumm, C.K. and Vogels, G.D. (1990) Eur. J. Protistol. *25*, 369-380.
2. Broers, C.A.M., Stumm, C.K. and Vogels, G.D. (1989) in Biochemistry and Molecu lar Biology of "Anaerobic" Protozoa (Lloyd, D., Coombs, G.H. and Paget, T.A. eds.), pp. 22-31, Harwood Publishers, London.
3. Broers, C.A.M., Stumm, C.K. and Vogels, G.D. (1989) Forum Microbiol. *12*, 53 V113 (abstract).
4. Van Bruggen, J.J.A., Zwart, K.B., Van Assema, R.M., Stumm, C.K. and Vogels, G.D. (1984) Arch. Microbiol. *139*, 1-7.
5. Stumm, C.K. and Zwart, K.B. (1986) Microbiol. Sci. *3*, 100-105.
6. Wagener, S. and Pfennig, N. (1987) Arch. Microbiol. *149*, 4-11.
7. Wagener, S., Bardele, C.F. and Pfennig, N. (1990) Arch. Microbiol. *153*, 496-501.
8. McInerney, M.J., Bryant, M.P. and Pfennig, N. (1979) Arch. Microbiol. *122*, 129-135.
9. Van Bruggen, J.J.A., Van Rens, G.L.M., Geertman, E.J.M., Zwart, K.B., Stumm, C.K. and Vogels, G.D. (1988) J. Protozool. *35*, 20-23.
10. Balch, W.E., Fox, G.F., Magrum, L.J., Woese, C.R. and Wolfe, R.s. (1979) Microbiol. Rev. *43*, 260-296.
11. Doddema, H.J. and Vogels, G.D. (1978) Appl. Environ. Microbiol. *36*,752-754.
12. Hutten, T.J., De Jong, M.H., Peeters, B.P.H., Van der Drift, C. and Vogels, G.D. (1981) J. Bacteriol. *145*, 27-34.
13. Goosen, N.K., Van der Drift, C., Stumm, C.K. and Vogels, G.D. (1990) FEMS Microbiol. Lett. *69*, 171-176.
14. Burnstone, M.S. (1961) J. Histochem. Cytochem. *9*, 59-65.
15. Seligman, A.M., Karnovski, M.J., Wasserkrug, H.L. and Hanker, J.S. (1968) J. Cell. Biol. 1-14.
16. Goosen, N.K., Wagener, S. and Stumm, C.K. (1990) Arch. Microbiol. *153*, 187-192.
17. Fenchel, T. and Finlay, B.J. (1990) FEMS Microbiol. Ecol. *74*, 269-276.
18. Fenchel, T. and Finlay, B.J. (1991) Eur. J. Protistol. *38*, 22-28.
19. Finlay, B.J. and Fenchel, T. (1991) FEMS Microbiol. Ecol. *85*, 169-180.
20. Paget, T.a. and Lloyd, D. (1990) Mol. Biochem. Parasitol. *41*, 65-72.
21. Gregory, E.M., Goscin, S.A. and Fridovich, I. (1974) J. Bacteriol. *117*, 456-460.
22. Mack, S.R. and Müller, M. (1978) J. Parasitol. *64*, 927-929.
23. Gregory, E.M. and Fridovich, I. (1973) J. Bacteriol. *114*, 543-548.
24. Müller, M. (1988) Ann. Rev. Microbiol. *42*, 465-488.
25. Fenchel, T. and Finlay, B.J. (1990) J. Gen. Microbiol. *136*, 1953-1959.
26. Fenchel, T. and Finlay, B.J. (1991) J. Protozool. *38*, 18-22.
27. Fulton, C. (1970) in Methods in Cell Physiology (Prescott, D.M., ed.), Vol. 4, pp. 341-476, Academic Press, New York.
28. Hollande, A. (1942) Arch. Zool. Expér. et Gén. *83*, 1-268.

CHAPTER 3

MONOXENIC CULTIVATION OF THE ANAEROBIC AMOEBO-FLAGELLATE *PSALTERIOMONAS LANTERNA* AND ISOLATION OF THE METHANOGENIC ENDOSYMBIONT

FEMS Microbiology Letters 92 (1992) 115–118

Published by Elsevier

FEMSLE 04834

Monoxenic cultivation of the anaerobic amoeboflagellate *Psalteriomonas lanterna* and isolation of the methanogenic endosymbiont

Cees A.M. Broers, Claudius K. Stumm and Godfried D. Vogels

Department of Microbiology and Evolutionary Biology, Faculty of Science, University of Nijmegen, Nijmegen, Netherlands

Received 25 November 1991
Revision received 29 January 1992
Accepted 30 January 1992

Key words: Endosymbiosis; *Methanobacterium formicicum*; *Psalteriomonas lanterna*; Monoxenic culture

1. SUMMARY

The endosymbiont of the anaerobic amoeboflagellate *Psalteriomonas lanterna* was isolated and identified as *Methanobacterium formicicum*, a H_2/CO_2- and formate-utilizing methanogen. A monoxenic culture of *P. lanterna* was established in a complex liquid medium with *Pseudomonas fluorescens* as food bacterium.

2. INTRODUCTION

Free-living anaerobic protozoa are known to harbour endosymbiotic methanogenic bacteria [1,2]. The endosymbionts from some freshwater species could be isolated and identified as *Methanobacterium formicicum*, a H_2/CO_2- and formate-utilizing methanogen [3–5]. The methanogenic bacteria were found to be more or less closely associated with microbodies, which were identified as hydrogenosomes using a cytochemical technique [6,7]. An interspecies hydrogen transfer is believed to be the physiological background of the endosymbiosis [4,8]. An endosymbiotic association with methanogenic bacteria was found also in *Psalteriomonas lanterna*, an amoeboflagellate assigned to the class of Heterolobosea [9,10]. The endosymbionts are closely associated with microbodies, presumably hydrogenosomes, located as a distinct aggregate in the centre of the cell [9,10]. *P. lanterna* is an interesting organism for phylogenetic, physiological and biochemical studies, because there is an intriguing possibility that the organism has both hydrogenosomes and mitochondria [9,10]. However, a detailed study in this respect is hampered by the lack of an axenic culture of *P. lanterna*.

In this paper we report on the monoxenic culture of *P. lanterna* and the isolation of the methanogenic endosymbiont.

Correspondence to C.A.M. Broers. Present address: Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands.

3 MATERIALS AND METHODS

3.1 Polyxenic cultivation of P. lanterna

Psalteriomonas lanterna cells containing methanogenic endosymbionts were cultured anaerobically on a mixed bacterial flora as described previously [10]. Symbiont free amoeboflaggellates [9,10] were grown in the same medium, with a refreshment of the gas phase (99% N_2, 1% O_2) twice a week.

3.2 Isolation of the methanogenic endosymbiont

An isolation method was used as previously applied for the isolation of endosymbiotic methanogens from *Plagiopyla nasuta* [3]. Washed flagellates were brought into liquid complex medium [4], supplemented with 1000 U/ml penicillin and 100 μg/ml streptomycin. The flagellate cells lysed immediately releasing the methanogenic bacteria. Pure cultures of methanogenic bacteria were obtained by the method of Van Bruggen et al. [4]. Methane was measured according to Hutten et al. [11].

3.3 Preparation of media for monoxenic cultures

For monoxenic cultivation a basal medium was used with the following composition: 2 mM Na/K phosphate buffer (pH 7), 0.1% (w/v) Pfennig's metal solution [12], 0.1 mM $MgSO_4$, 0.1% (w/v) glutathione. The medium was distributed in 10-ml quantities in 16 × 150-mm Hungate tubes (Bellco Glass Inc., Vineland, NJ). The tubes were closed with butyl rubber stoppers and screw caps, evacuated and gassed with N_2 or N_2/CO_2 95/5 (130 kPa). After autoclaving, 0.1 ml filter-sterilized 0.3 M $NaHCO_3$ and 0.3 ml heat-inactivated fetal calf serum (Gibco) was added to the basal medium by using a hypodermic syringe.

3.4 Procedure to obtain monoxenic cultures

Symbiont-free flagellates from polyxenic cultures in an early stationary growth phase were harvested by an electromigration technique [18]. The cells were washed twice with sterile anaerobic 10 mM phosphate buffer (pH 6.8), supplemented with 0.2 mM cystein HCl. The sedimented cells were resuspended in 0.2 ml of the same buffer and transferred with a syringe to culture tubes. Each tube received 5×10^4 cells. The tubes contained the basal medium supplemented with 0.2% (w/v) malt extract and 0.2% (w/v) yeast extract (MYG-medium). Bacteria were eliminated by the addition of antibiotics (penicillin 2000 U/ml, streptomycin 50 μg/ml) to the medium. The culture tubes were incubated in a slanted position at 24°C. The cells were transferred every 3–5 days by inoculation of 1-ml samples to fresh MYG medium containing the antibiotic mix. After four transfers the cultures were free of living bacteria. Growth of the flagellate ceased in this stage but could be restored by the addition of 0.1 ml of a suspension (10^{10} cells/ml) of *Pseudomonas fluorescens*, an isolate from the polyxenic culture. The bacteria were pregrown on agar plates with 1.5% proteose peptone. After the establishment of a monoxenic culture, *P. lanterna* cells were grown in 50-ml serum bottles with 20 ml MYG medium supplemented with 10^8 *P. fluorescens* cells per ml and were subcultured every 3–4 weeks by the transfer of 1 ml inoculum to fresh medium.

3.5 Microscopy

Growth of the flagellates was followed by a direct examination in the culture vessels by using an inverted microscope (Labovert Leitz). Flagellate cell densities were determined by counting cells in 0.1 ml samples fixed with Lugol. Electron microscopy was performed as described previously [10].

4 RESULTS

4.1 Characterization of the isolated methanogenic endosymbiont

In *P. lanterna* the methanogenic bacteria and microbodies are organized in a globular structure, the bacteria–microbody complex (BMC), which has been described elsewhere [10]. The complex contained usually about 200 methanogens, which showed morphological similarities with *Methanobacterium formicicum* (Fig. 1).

The isolated methanogen was a slender, nonmotile rod (2–6 × 0.4 μm) often occurring as chains. On solid agar medium, convex, whitish-

yellow colonies were found with a smooth surface and an entire margin. The bacterium was able to grow on H_2/CO_2 and formate. No growth was observed on acetate, methanol, methylamine, glucose or pyruvate under a gas phase of 80% N_2 and 20% CO_2. Growth of the isolate was observed at temperatures ranging from 15°C to 45°C with an optimum at 37°C. When incubated in mineral liquid medium [4] with formate at 37°C, a generation time of 12 h was found. The above-mentioned characteristics indicate that most probably the isolate is *Methanobacterium formicicum*, although further studies are required.

4.2. Monoxenic cultivation of P. lanterna

The establishment of a monoxenic culture using the basal medium with various food bacteria was unsuccessful. No growth occurred either when *P. lanterna* was transferred to the medium used for growth of monoxenic *Trimyema compressum*, with *Bacteroides* sp. strain WoCb as food bacterium [13,14]. However, growth of *P. lanterna* occurred in MYG medium supplemented with *pseudomonas fluorescens*. Fetal calf serum was indispensible and growth of the food bacterium was suppressed by maintaining strictly anaerobic conditions or by the addition of 500 U/ml penicillin and 50 μg/ml streptomycin to the medium. The living *P. fluorescens* bacteria could not be omitted, nor be replaced by dead bacteria (heat-killed for 1 h at 65°C, or autoclaved). Purity of the monoxenic cultures was tested regularly by microscopical observation and by the inoculation

Fig. 1. Detail of the bacteria–microbody complex showing a longitudinal section of a methanogenic endosymbiont (B). Electron-dense material inside the bacterial cell has been segregated into two parts and consists probably of DNA. H, hydrogenosomes. Bar, 0.5 μm.

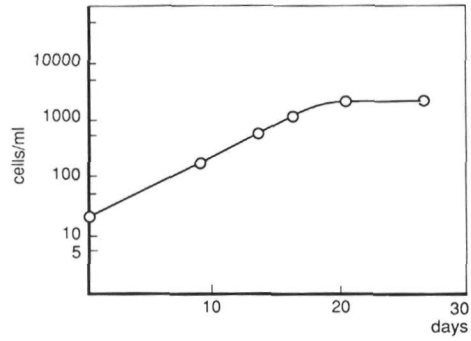


Fig. 2. Growth curve of symbiont-free *P. lanterna* cells in MYG medium under an atmosphere of N_2/CO_2 (95/5). The flagellates were fed with 10^8 cells/ml of *P. fluorescens*.

of culture samples to tryptone soya broth agar plates which were incubated under aerobic as well as anaerobic conditions.

Flagellates growing in the MYG medium together with *P. fluorescens* reached densities of 2400 cells/ml, with doubling times of 2–3 days (Fig. 2). Replacement of the yeast extract by 0.2% trypticase resulted in an increased cell density of 4000 cells/ml. The cultures produced H_2 (not quantified). No methane production could be measured, and methanogenic bacteria could not be detected using epifluorescence microscopy.

5. DISCUSSION

The isolate has the properties of *Methanobacterium formicicum*. The same bacterium was isolated earlier from several other protozoa [3–5] and seems to be a common endosymbiont of freshwater anaerobic protozoa. In *P. lanterna* the methanogens are closely associated with microbodies which could be identified as hydrogenosomes (manuscript in preparation). The localization of hydrogenase in the microbodies together with the substrate requirements of the endosymbionts makes the hypothesis of an interspecies hydrogen transfer plausible [4]. Both the host and the endosymbiont may profit from the relation-

ship. The benefit of the association for both partners has been discussed elsewhere [1,4,14–16].

It is difficult to obtain axenic cultures of amoeboflagellates [17]. Therefore, the establishment of a monoxenic culture of *P. lanterna* is a step forward to cultivate the amoeboflagellate under defined conditions and may later facilitate axenic cultivation In contrast to the symbiont-free amoeboflagellates in polyxenic cultures [9] the monoxenically cultivated *P lanterna* cells were able to grow under strictly anaerobic conditions. The absence of H_2-producing bacteria in the monoxenic cultures may explain this observation. Unlike the food bacteria in the polyxenic cultures, *P. fluorescens* does not produce H_2. Only the amoeboflagellate produces H_2, but probably in such a small quantity that the inhibitory effects do not occur at a population density of 4000 cells/ml. However, the maximum cell density and the growth rate of the monoxenic cultures were reduced as compared to the polyxenic grown cells, which reach densities of 12×10^3 cells/ml at a growth rate of 18 h [9,10].

REFERENCES

[1] Fenchel, T and Finlay, B J (1991) Eur J Protistol 26, 201–215

[2] Van Bruggen, J J A, Stumm, C K and Vogels, G D (1983) Arch Microbiol 136, 89–95

[3] Goosen, N K, Horemans, A M C, Hillebrand, S J W, Stumm, C K and Vogels, G D (1988) Arch Microbiol 150, 165–170

[4] Van Bruggen, J J A, Zwart, K B, van Assema, R M, Stumm, C K and Vogels, G D (1984) Arch Microbiol 139, 1–7

[5] Van Bruggen, J J A, van Rens, G L M, Geertman, E J M, Zwart, K B, Stumm, C K and Vogels, G D (1988) J Protozool 35, 20–23

[6] Finlay, B J and Fenchel, T (1989) FEMS Microbiol Lett 65, 311–314

[7] Zwart, K B, Goosen, N K, van Schijndel, M W, Broers, C A M, Stumm, C K and Vogels, G D (1988) J Gen Microbiol 134, 2165–2170

[8] Stumm, C K and Zwart, K B (1986) Microbiol Sci 3, 100–105

[9] Broers, C A M., Stumm, C K and Vogels, G D (1989) In Biochemistry and Molecular Biology of "Anaerobic" Protozoa (Lloyd, D, Coombs, G H and Paget, T A, Eds), Harwood Academic Publishers, London, pp 22–31

[10] Broers, C A M, Brugerolle, G, Stumm, C K and Vogels, G D (1990) Eur J Protistol 25, 369–380

[11] Hutten, T J, de Jong, M H, Peeters, B P H, van der Drift, C and Vogels, G D (1981) J Bacteriol 145, 27–34

[12] McInerney, M J, Bryant, M P and Pfennig, N (1979) Arch Microbiol 122, 129–135.

[13] Goosen, N K, Wagener, S and Stumm, C K (1990) Arch Microbiol 153, 187–192

[14] Wagener, S and Pfennig, N (1987) Arch Microbiol 149, 4–11

[15] Fenchel, T and Finlay, B J (1991) J Protozool 38, 18–22

[16] Fenchel, T and Finlay, B J (1991) J Protozool 38, 22–28

[17] Dexter Dyer, B (1990) In Handbook of Protoctista (Margulis, L, Corliss, J O, Melkonian, M and Chapman, D J Eds), Jones and Bartlett Publishers, Boston, MA, pp 186–190

[18] Broers, C A M, Molhuizen, H O F, Stumm, C K and Vogels, G D (1991) J Microbiol Methods 14, 217–220

CHAPTER 4

HYDROGENOSOMES AND PRESUMED MITOCHONDRIA IN THE ANAEROBIC AMOEBOFLAGELLATE *PSALTERIOMONAS LANTERNA*

Cees A.M. Broers. Rianne v. d. Wiel, Claudius K. Stumm and G.D. Vogels

Submitted for publication in Biosystems

SUMMARY

The anaerobic amoeboflagellate *Psalteriomonas lanterna* possesses two types of cytoplasmic organelles: microbodies and presumed modified mitochondria. The microbodies could be identified as hydrogenosomes by the demonstration of hydrogenase. The presumed mitochondria were found to take up rhodamine 123 and they probably contain DNA. Cytochromes could not be detected and cytochemical tests for succinate dehydrogenase and citrate (isocitrate) hydro-lyase were negative. The presence of the two organelles within *P. lanterna* favours the hypothesis of an anaerobic prokaryotic origin for the hydrogenosomes.

INTRODUCTION

Many protists live in anaerobic environments, they lack mitochondria and perform a fermentative metabolism (21). Some of them have an entirely cytosolic metabolism but in others the metabolism is partly compartmentalized in hydrogenosomes (20, 21). Hydrogenosomes are organelles surrounded by one or two unit membranes and contain apparently no DNA (20, 21). They are regarded as the anaerobic equivalents of mitochondria. In hydrogenosomes pyruvate is oxidized under the formation of acetate, CO_2 and H_2, yielding ATP by substrate level phosphorylation. The organelle is well studied from parasitic flagellates, rumen ciliates and fungi (20, 21). Recently, hydrogenosomes were demonstrated in free-living ciliates, by using immunofluorescent and cytochemical stain techniques (3, 10, 11, 29). The origin of the hydrogenosomes is obscure; it is argued that they are derived from mitochondria (6) or from endosymbiotic anaerobic bacteria (21). Alternatively, a common progenitor of both mitochondria and hydrogenosomes has been proposed (19).

Hitherto, organisms containing hydrogenosomes were found to be devoid of mitochondria. In this respect, the amoeboflagellate *Psalteriomonas lanterna* may occupy an unique position. The organism has microbodies which are probably hydrogenosomes as indirectly inferred from their association with methanogenic endosymbionts. Furthermore, a second type of organelle is present, showing some ultrastructural similarities with mitochondria (1, 2).

In this paper more information concerning both organelles is presented, and some evolutionary aspects are discussed.

MATERIAL AND METHODS

Cultivation and harvesting

Psalteriomonas lanterna was grown in phosphate-buffered medium containing a mixed population of food bacteria, as previously described (2). Symbiontfree flagellates were maintained under an atmosphere of 1% O_2 with a refreshment of the gasphase twice a week. Flagellate cells from up to 300 cultures (20 ml each) were harvested and simultaneously separated from the food bacteria by applying an electromigration technique (4) or by centrifugal elutriation by using a Curamé IPS-4 centrifuge at 1 000 g with a flow rate of 20 ml/min. Prior to the experiments the cells were centrifuged at 800 g for 3 min and washed twice with anaerobic 7 mM phosphate buffer.

Characterization of the microbodies

The microbodies were studied by means of a cytochemical technique for the localization of hydrogenase activity (28), with H_2 as substrate and the tetrazolium salt BSPT [2-(2'-benzothiazolyl)-5-styryl-3-(4'phthalhydrazidyl)-tetrazolium chloride] as electron acceptor. The reaction was followed under the light microscope.

Alternatively, fixed cells were incubated with a polyclonal antiserum (rabbit) against hydrogenosomal hydrogenase and subsequently treated with a FITC-conjugated secondary antibody as described previously (3). The localization of the immunofluorescent staining was detected using a MRS/Lasersharp confocal microscope (BioRad).

Characterization of the mitochondrion-like organelles

The presence of cytochromes in the cells was investigated by optical redox difference spectroscopy of a dense flagellate suspension by using a Hitachi U-3200 spectrophotometer.

The fluorescent dye rhodamine 123 (Kodak, Eastman Lab, Rochester, N.Y.) was used to stain specifically mitochondria in living cells (16, 17). To a flagellate culture, which was pregrown for 48 h under an atmosphere of 2% O_2, rhodamine 123 was added to give a final concentration of 10 μg/ml. After an incubation for 30 minutes at room temperature, the cells were harvested by gentle centrifugation (3 min, 800 g) and washed twice with 7 mM phosphate buffer pH 6.8, saturated with 98% N_2 and 2% O_2. Wet mount preparations were viewed by using a confocal microscope (MRC/Lasersharp, BioRad) at 485 nm excitation.

To detect mitochondrial DNA in *P. lanterna*, unfixed or fixed cells (in 0.5% glutaraldehyde) were treated with 1 μg/ml 4',6-diamidine-2-phenylindole (DAPI), a fluorochrome preferentially binding to A+T rich DNA (14). Stained cells were examined by epifluorescence microscopy at 360 nm.

Succinate dehydrogenase activity (SDH) was investigated by using the method of Kalina et

al. (1972). Fixed cells (2% paraformaldehyde) were incubated for 1-2 h in a reaction mixture containing Tris HCl pH 7.2 (50 mM), BSPT (0.25 mg/ml), sucrose (0.25 M) and Na-succinate (1 M). In control experiments malate (50 mM) or KCN (10 mM) was added to the reaction mixture or Na-succinate was omitted. Succinate dehydrogenase activity was also detected by the reduction of ferricyanide according to the method of Ogawa et al. (22).

Citrate (isocitrate) hydro-lyase was localized according to Pearse (1972), with BSPT as electron acceptor and cis-aconite as substrate. Unfixed as well as formaldehyde (1%)-fixed cells were used in this experiment.

For the cytochemical localization of enzymes, cells of *Paramecium caudatum* were used as positive control in the experiments. All reactions were followed under the light microscope.

RESULTS AND DISCUSSION

In a previous study, *P. lanterna* was found to contain two types of cytoplasmic organelles, microbodies and presumably modified mitochondria (1, 2). Both organelles differ morphologically in size (e.g. compare Fig. 4, 6 and 7), and in the association with rough endoplasmic reticulum (RER) or methanogenic bacteria (2) (Fig. 1-4).

The microbodies were surrounded by two unit membranes (Fig. 2). The organelles were highly osmiphilic. They were exclusively found in the centre of the cell where they formed a spherical cluster together with methanogenic endosymbionts (1, 2). Continued cultivation of P. lanterna under low oxygen tensions caused a loss of the methanogenic bacteria, but did not affect the clustering of the microbodies, nor did it result in an association with RER.

Recently, the methanogenic endosymbionts of *P. lanterna* were found to utilize H_2 (5). This substrate requirement was an indication of a hydrogenosomal nature of the microbodies. The presence of hydrogenase in the microbodies was tested by using the cytochemical BSPT-staining. Applying this method, hydrogenase activity could be localized in the cluster of microbodies and endosymbionts (Fig. 5). The positive reaction was assigned to the microbodies and not to the methanogens since the same results were obtained with endosymbiont-free *P. lanterna* cells (results not shown). Furthermore, methanogenic endosymbionts, which also have a hydrogenase, were found to show a negative reaction after staining for hydrogenase activity in anaerobic ciliates (11).

The presence of hydrogenase in the microbodies was confirmed by indirect immunofluorescence, showing cross reactivity of hydrogenosomal hydrogenase antiserum with the microbodies of *P. lanterna* (Fig. 6). These results strongly indicate that the microbodies are hydrogenosomes. However, attempts failed to localize the hydrogenosomal enzyme malate dehydrogenase (decarboxylating) (13). The substrate requirements of the endosymbionts together with the localization of hydrogenase validate the hypothesis of an interspecies

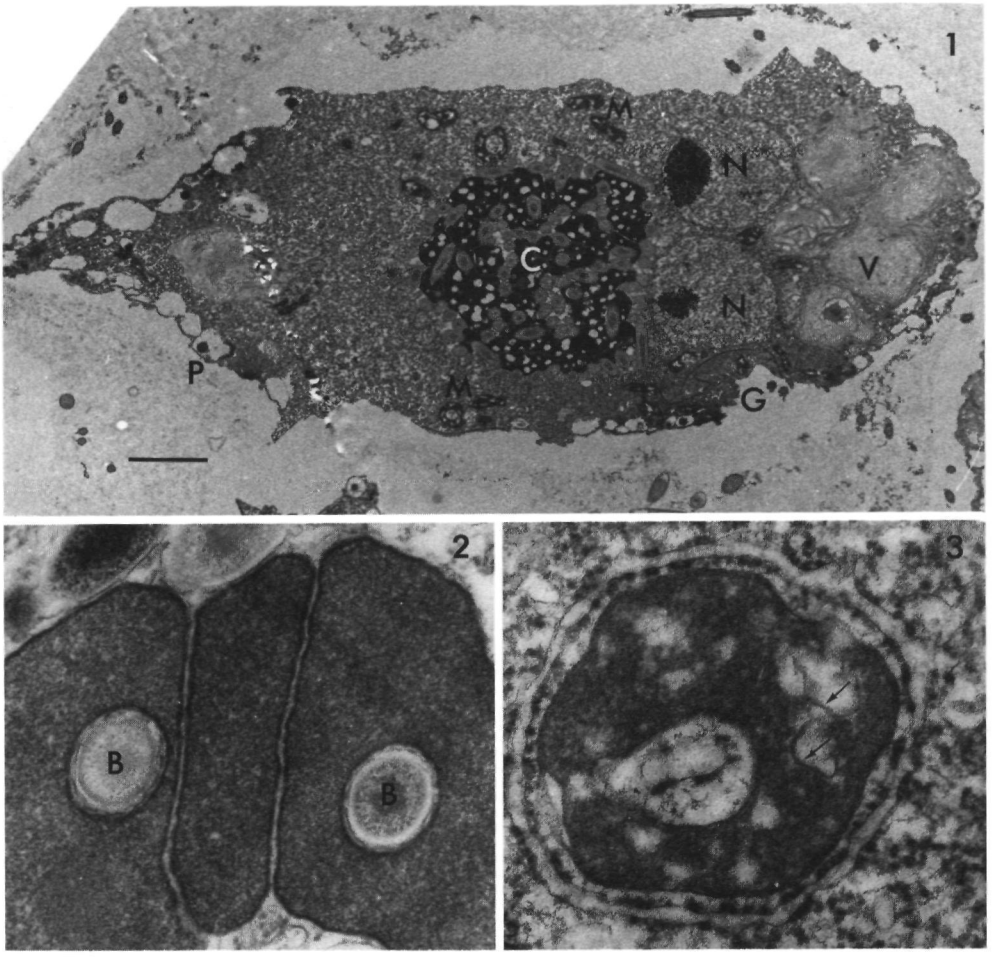


Fig. 1. Transmission electron micrograph of a longitudinal section of the flagellate stage of *P. lanterna* showing the general organization of the organism. In the centre of the cell a cluster (C) of hydrogenosomes and methanogenic bacteria are located. The presumed mitochondria (M), some of them cup-shaped, are found dispersed in the cytoplasm. The matrix of the hydrogenosomes and the presumed mitochondria often contain lancunae (not covered by a membrane), but this is probably a fixation artifact. G = anterior part of the groove with associated microtubular ribbons; N = nucleus; P = pigment granule; V = food vacuole. Scale bar = 2 μm.

Fig. 2. Part of the cluster of hydrogenosomes and methanogenic bacteria. Some bacteria (B) are completely enclosed by the microbodies. Scale bar = 0.5 μm.

Fig. 3. An organel presumed to be a modified mitochondrion. The cup-shaped organel is surrounded by RER. Some internal membrane structures are visible (arrows).

hydrogen transfer as the physiological background of the endosymbiosis (26).

In contrast to the hydrogenosomes, which are clustered, the modified mitochondria are spread throughout the cytoplasm (Fig. 1, 4). Like the mitochondria of all heterolobosean amoeboflagellates (23) the organelles are surrounded with RER (2; Fig. 3). However, in *P. lanterna* they lack cristae, though sometimes invaginations of the inner membrane were observed (Fig. 3). Cultivation of the amoeboflagellate under low oxygen tensions did not result in an increase of the infolding. Furthermore, under the culture conditions applied, the organelles were found to be cytochrome oxidase negative (1).

The presumed modified mitochondria, as identified by using phase contrast microscopy (Fig. 4), were shown to take up rhodamine 123 (Fig. 7), a dye selectively staining mitochondria (16, 17). In cells cultured under strictly anaerobic conditions, the fluorescent staining was less bright.

Similar results were obtained using the dyes 3'3-dihexyloxacarboyanin (1) and dimethyl amino stipyl-pyridylethyl lodide (not shown). The positive reaction indicates the existence of a transmembrane potential in the organelles and therefore some physiological activity. However, spectral analysis on symbiont-free whole flagellate cells did not reveal the presence of cytochromes in this organism. Probably more sensitive techniques are required to demonstrate cytochromes.

Mitochondria are the only organelles, together with chloroplasts, which contain extranuclear DNA. Staining of the flagellate cell with DAPI showed fluorescent particles in the cytoplasm (Fig. 8). Molecular biological studies are undertaken now to detect and characterize the DNA which is probably present in the modified mitochondria. Tests to localize the mitochondrial enzymes succinate dehydrogenase and citrate (isocitrate) hydrolyase cytochemically were negative. As found for the mitochondria of the anaerobic protist *Blastocystis hominis* (28), complete enzyme systems might be absent in the organelles of P. lanterna.

From the results reported here it can be concluded that *P. lanterna* has two distinct types of organelles with hydrogenosomal and mitochondrial properties, respectively. The ultrastructural and functional differences between both organelles as well as their simultaneous and consistent presence in the flagellates make it not plausible that they originate from each other. Furthermore, the presence of a double membrane precludes a peroxisomal origin for the hydrogenosomes. These findings affirm the hypothesis of a symbiotic origin from anaerobic bacteria for the hydrogenosomes (21).

The presence of both hydrogenosomes and mitochondria-like organelles in P. lanterna raises the question whether the anaerobic style of life of this organism is a primary feature or a secondary adaptation. A primary feature would imply that *P. lanterna* is a direct descendent of amitochondrial protists living in periods of early eukaryotic evolution when the earth atmosphere was largerly oxygen-free. The primitiveness of *P. lanterna* and other heterolobosean

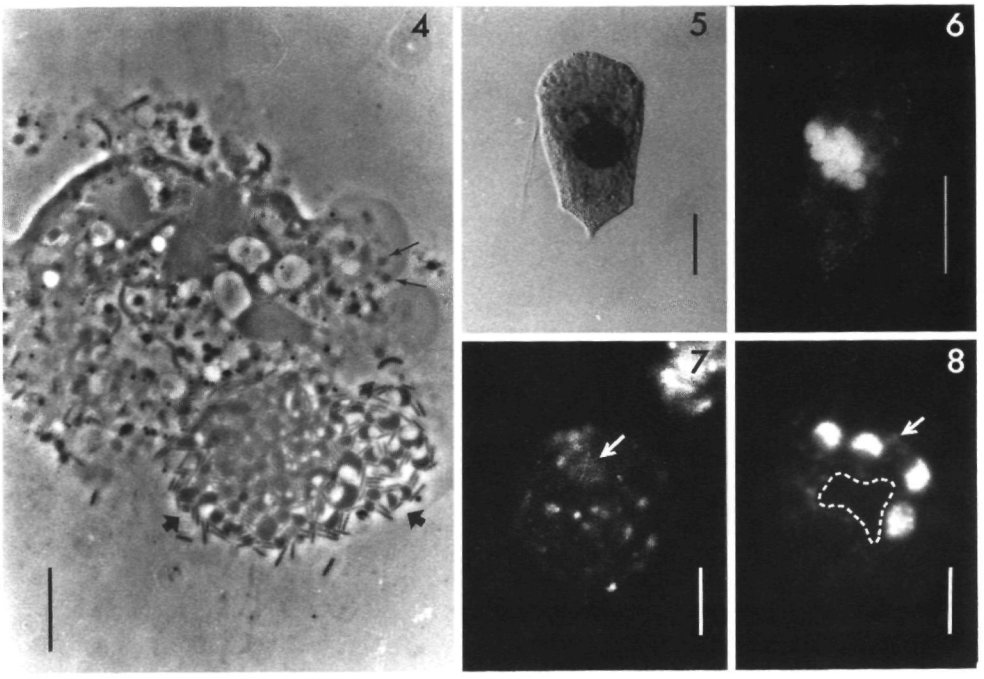


Fig. 4. Squashed flagellate cell, showing some of the presumed mitochondria (thin arrows) and the cluster of hydrogenosomes and methanogenic endosymbionts (thick arrows). Phase contrast light microscopy. Scale bar = 10 μm.

Fig. 5. Light micrograph of a cell stained for hydrogenase activity using BSPT. A positive reaction is found in the cluster of microbodies and methanogenic bacteria. Scale bar = 10 μm.

Fig. 6. Light micrograph of a symbiont-free flagellate cell, immunocytochemically labelled with hydrogenase antibodies. Scale bar = 10 μm.

Fig. 7. Optical section of a symbiont-free cell treated with rhodamine 123. The modified mitochondria are selectively stained. The cluster of hydrogenosomes (arrow) shows some weak autofluorescence. Scale bar = 10 μm.

Fig. 8. Cell stained with DAPI, showing the four nuclei and small particles in the cytoplasm which may represent mitochondrial DNA. The larger fluorescent particles (->) are probably freshly ingested bacteria, aggregated in food vacuoles. The cluster of hydrogenosomes is located in the area circumscribed by the broken line; they contain no detectable DNA. Scale bar = 10 μm.

amoeboflagellates might be indicated by the intranuclear mitosis and by the absence of a Golgi complex (7). Furthermore, the ancestral nature of the Heterolobosea was confirmed by 16S rRNA studies of *Naegleria*, which place the organism early in the phylogenetic tree (8, 25).

Assuming also tetrakonty as an ancestral property (15), an amitochondrial psalteriomonad-like organism seems to be a probable candidate as ancestor for the aerobic amoeboflagellates, e.g. *Tetramitus rostratus* and *Naegleria*. However, some reservation must be made since the position of *Naegleria* in the phylogenetic tree, apart from a misplacement (27), indicates that the Heterolobosea emerged after the acquisition of mitochondria by the eukaryotic cell. From this point of view the presence of hydrogenosomes in *P. lanterna* represents a secondary adaptation to anaerobiosis, and the modified mitochondria should be considered as vestigial organelles rather than mitochondria in an early stage of development.

REFERENCES

1. Broers, C.A.M., Stumm, C.K. and Vogels, G.D. 1989, A heterolobose amoeboflagellate associated with methanogenic bacteria, in: Biochemistry and Physiology of "Anaerobic" Protozoa. D. Lloyd, G.H. Coombs and T.A. Paget (eds.) (Harwood Publishers, London), pp. 22-31.
2. Broers, C.A.M., Brugerolle, G., Stumm, C.K. and Vogels, G.D. 1990, *Psalteriomonas lanterna* gen. nov., sp. nov., a free-living amoeboflagellate isolated from freshwater anaerobic sediments. Europ. J. Protistol. *25*, 369-380.
3. Broers, C.A.M., Stumm, C.K. and Vogels, G.D. 1991, Axenic cultivation of the anaerobic free-living ciliate *Trimyema compressum*. J. Protozool. *38*, 507-511.
4. Broers, C.A.M., Molhuizen, H.O.F., Stumm, C.K. and Vogels, G.D. 1992, An electromigration technique, applied for the concentration of anaerobic protozoa from mass cultures. J. Microbiol. Meth. *14*, 217-220.
5. Broers, C.A.M., Stumm, C.K. and Vogels, G.D. 1992, Monoxenic cultivation of the anaerobic amoeboflagellate *Psalteriomonas lanterna* and isolation of the methanogenic endosymbiont. FEMS Microbiol. Lett. *92*, 115-118.
6. Cavalier-Smith, T. 1987, The simultaneous symbiotic origin of mitochondria, chloroplasts and microbodies. Ann. NY Acad.Sci. 503, 55-72.
7. Cavalier-Smith T. (1991). Archamoebae: the ancestral eukaryotes? Biosystems *25*, 25-38.
8. Clark, G.C. 1990, Genome structure and evolution of *Naegleria* and its relatives. J. Protozool. *37*, 2S-6S.
9. Fenchel, T. and Patterson, D.J. 1986, *Percolomonas cosmopolitus* (Ruinen) n. gen., a new type of filter feeding flagellate from marine plankton. J. Mar. Biol. Ass. U.K. *66*, 465-482.
10. .Fenchel, T. and Finlay, B.J. 1991, The biology of free-living anaerobic ciliates. Europ. J. Protistol. *26*, 201-205.
11. Finlay, B.J. and Fenchel, T. 1989, Hydrogenosomes in some anaerobic protozoa resemble mitochondria. FEMS Microbiol. Lett. *65*, 311-314.
12. Fulton, C. 1970, Amoeboflagellates as research partners. in: Methods in Cell Physiology. D.M. Prescott (ed.), vol. 4 (Academic Press, New York), pp. 341-476.
13. Goosen, N.K., Broers, C.A.M., Hombergen, E.-J., Stumm, C.K. and Vogels, G.D. 1990, Effect of fixation on activity and cytochemistry of hydrogenosomal enzymes in *Trichomonas vaginalis*. J. Gen. Microbiol. *136*, 2189-2193.
14. Hajduk, S.L. 1976, Demonstration of kinetoplast DNA in dyskinetoplastic strains of *Trypanosoma equiperdum*. Science *191*, 858-859.
15. Johnson, A. 1990, Phylogeny and evolution of protozoa. Zool. Sci. *7*, 179-188.
16. Johnson, L.V., Walsh, M.L. and Chen, L.B. 1980, Localization of mitochondria in living cells with rhodamine 123. Proc. Natl. Acad. Sci. USA *77*, 990-994.
17. Johnson, L.V., Walsh, M.L., Bockus, B.J. and Chen, L.B. 1981, Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J. Cell Biol. *88*, 526-535.

18. Kalina, M., Plapinger, R.E., Hoshino, Y. and Seligman, A.M. 1972, Nonosmiophilic tetrazolium salt that yield osmiophilic formazans for ultrastructural localization of dehydrogenase activity. J. Histochem. Cytochem. *20*, 685-695.
19. Lahti, C.J. and Johnson, P.J. 1991, *Trichomonas vaginalis* hydrogenosomal proteins are synthesized on free polyribosomes and may undergo processing upon maturation. Mol. Biochem. Parasitol. *46*, 307-310.
20. Lindmark, D.G., Eckenrode, B.L., Halberg, L.A. and Dinbergs, I.D. 1989, Carbohydrate, energy and hydrogenosomal metabolism of *Tritrichomonas foetus* and *Trichomonas vaginalis*. J. Protozool. *36*, 214-216.
21. Müller, M. 1988, Energy metabolism of protozoa without mitochondria. Ann. Rev. Microbiol. *42*, 465-488.
22. Ogawa, K., Saito, T. and Mayahara, H. 1968, The site of ferricyanide reduction by reductases within mitochondria as studied by electron microscopy. J. Histochem. Cytochem. *16*, 49-57.
23. Page, F.C. and Blanton, R.L. 1985, The Heterolobosea (Sarcodina: rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). Protistologica *21*, 121-132.
24. Pearse, A.G.E. 1972, Histochemistry. vol. 2. Churchill Livingstone, London.
25. Sogin, M.L. 1989, Evolution of eukaryotic microorganisms and their small subunit ribosomal RNAs. Amer. Zool. *29*, 487-499.
26. Van Bruggen, J.J.A., Zwart, K.B., Van Assema, R.M., Stumm, C.K. and Vogels, G.D. 1984, *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch. Microbiol. *139*, 1-7.
27. Wolters, J. 1991, The troublesome parasites - molecular and morphological evidence that Apicomplexa belong to the dinoflagellate-ciliate clade. Biosyst. *25*, 75-83.
28. Zierdt, C.H., Donnolley, C.T., Muller, J. and Constantopoulos, G. 1988, Biochemical and ultrastructural study of *Blastocystis hominis*. J. Clin. Microbiol. *26*, 965-970.
29. Zwart,, K.B., Goosen N.K., Van Schijndel, M.W., Broers, C.A.M., Stumm, C.K. and Vogels, G.D. 1988, Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compressum*. J. Gen. microbiol. *134*, 2165-2170.

CHAPTER 5

SYMBIOTIC ASSOCIATION OF *PSALTERIOMONAS VULGARIS* N. SPEC. WITH *METHANOBACTERIUM FORMICICUM*

Cees.A.M. Broers, Henk H.M. Meyers, Joep C. Symens, Brugerolle G., Claudius K. Stumm and Godfried D. Vogels. (1992), Eur. J. Protistol., In Press.

SUMMARY

The free-living anaerobic flagellate *Psalteriomonas vulgaris* n. spec. is described. The organism has four flagella of equal length which arise immediately subapically to the anterior part of the cell, within the apex of the ventral groove. The ultrastructural organization of the mastigont system and the ventral groove show the characteristics of the genus *Psalteriomonas*. The cells harboured methanogenic endosymbionts which were associated with hydrogenosome-like organelles in which hydrogenase could be localized. The methanogenic bacteria were isolated and identified as *Methanobacterium formicicum*. After addition of 5% O_2 to the cultures, the cells lost the methanogenic endosymbionts.
P. vulgaris lacked cytochrome oxidase and catalase but contained superoxide dismutase.

INTRODUCTION

Most free-living anaerobic protozoa harbour methanogenic bacteria and microbodies. From the freshwater ciliates *Metopus striatus* and *Plagiopyla nasuta* and the giant amoeboflagellate *Pelomyxa palustris* an endosymbiotic methanogen could be isolated and identified as *Methanobacterium formicicum* [12, 23, 24]. The nutrient requirement of this methanogen, viz. H_2/CO_2 or formate, matches well with the recent demonstration of hydrogenase activity in the microbodies of anaerobic ciliates suggesting an intracellular hydrogen transfer [11, 27]. The microbodies are believed to be hydrogenosomes, which are redox organelles producing hydrogen [18]. This paper deals with similar structures in the amoeboflagellate *Psalteriomonas vulgaris*. Furthermore, the cultivation of the organism, the localization of hydrogenase and the isolation of the methanogenic endosymbionts are described.

MATERIAL AND METHODS

Isolation and cultivation of the flagellate

For the enrichment of the flagellate, a 2 liter bottle was filled with a sample of anoxic sediments from a ditch near Oisterwijk, The Netherlands, plus ditch water supplemented with 0.1 g proteose peptone and 0.1 g casein hydrolysate to stimulate growth of food bacteria. The bottle was closed with a butyl rubber stopper. After a few weeks, samples from this enrichment culture containing flagellates were transferred to 50-ml serum bottles with 20 ml medium consisting of 5 mM phosphate buffer pH 7, 0.01% (w/v) $Na_2S.3H_2O$ -

and 0.05% (w/v) proteose peptone. The medium was previously made anaerobic with N_2 (130 kPa) in the headspace by using standard techniques. The bottles were closed with butyl rubber stoppers.

The medium and the inoculation procedure were selective to obtain mono-cultures of the flagellate. The organism was routinely cultivated at 24°C in anaerobic medium with the following composition: 7 mM Na/K phosphate buffer pH 6.8, 0.1% (w/v) cystein-HCl, 1 ml/l Pfennig's metal solution [17] and 0.025% w/v yeast extract (FM medium). An undefined bacterial population was continuously present and served as food source. Growth of the amoeboflagellate was followed by means of an inverted microscope. For studies on the effect of oxygen, this gas was added to the headspace using a hypodermic syringe.

Harvesting

For analytical procedures, the flagellates were concentrated by gentle centrifugation (5 min, 800 x g). Subsequently, the cell suspension was layered over a 40% Percoll cushion prepared anaerobically in 7 mM phosphate buffer pH 6.8. The cells were centrifuged at 1500 x g for 15 min. The flagellates banded at the interface, whereas bacteria and debris sedimented as a pellet. The cells were collected with a Pasteur pipette and washed two times with anaerobic phosphate buffer (7 mM) pH 6.8 and 0.1 mM cystein-HCl.

Microscopical procedures

To determine flagellate numbers, the cells were counted in 0.1 ml samples fixed with Lugol's solution. For detection of methanogenic bacteria, epifluorescence microscopy was applied [6].

For scanning electron microscopy (SEM), cells were fixed according to Parducz [19] and adhered to coverslips coated with poly-L-lysine. After dehydration through ethanol and critical point drying the flagellates were coated with gold and examined with a JEOL scanning electron microscope (JSM-U3).

For transmission electron microscopy (TEM), cells were fixed for 30 min in an ice-cold mixture of 2% glutaraldehyde and 1% OsO_4 in 70 mM Na/K phosphate buffer pH 7.2. The cells were stained with 1% uranylacetate for 1 h and ultrathin sections were poststained with lead citrate. The sections were examined in a Philips EM 201 electron microscope.

Isolation of the methanogenic endosymbiont

About 100 flagellate cells were collected with a micropipette and washed 5 times with sterile anaerobic buffer (7 mM phosphate buffer pH 6.8 and 0.1 mM cystein-HCl). The flagellates and the last wash buffer (blank) were transferred with a hypodermic syringe into a 10 ml serum bottle, containing 2 ml complex medium for the enrichment of methan-

ogens[12], supplemented with 1000 U/ml penicillin and 100 μg/ml streptomycin. The subsequent isolation of the endosymbiont was according to the method of Van Bruggen et al. [22].

Indirect immunofluorescence

Hydrogenase was localized using polyclonal antiserum against hydrogenosomal hydrogenase and FITC-conjugated secondary antibodies (anti-rabbit immunoglobulines) as previously described [3]. The cells were incubated for 30 min at 37°C to facilitate a better reaction with the hydrogenase antiserum. The immunofluorescence was detected using a MCR-Lasersharp confocal microscope (BioRad).

Analytical procedures

Catalase activity was determined by transferring a concentrated cell suspension into a drop of 3% aqueous hydrogen peroxide. Superoxide dismutase (SOD) activity was traced in whole cells with riboflavine, methionine and nitro-blue tetrazolium salt in the reaction mixture [13].

Cytochrome oxidase activity was detected using the method of Burnstone [5], with n-phenyl-p-phenylenediamine and 8-amino-1,2,3,4-tetrahydroquinoline as coupler.

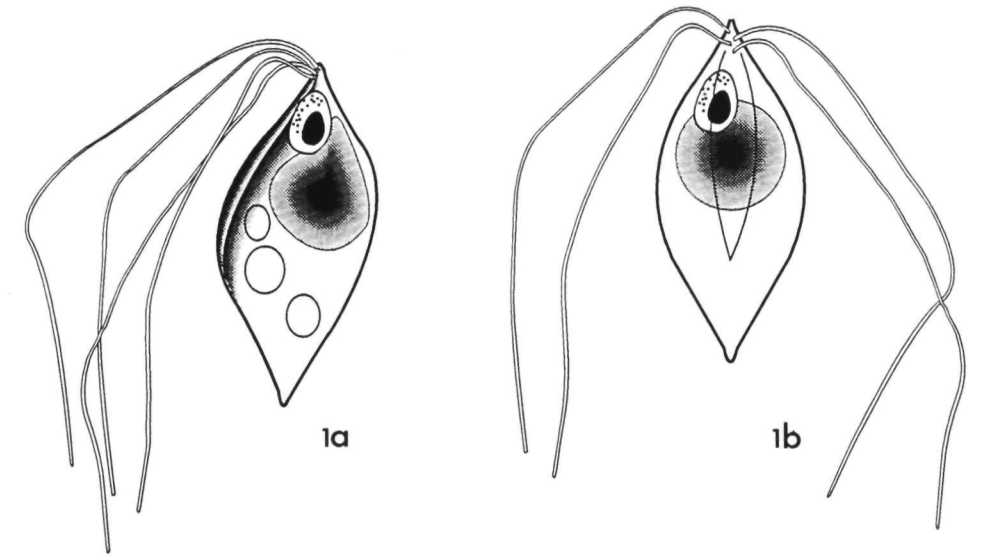
RESULTS

Monoculture

Microscopical inspection of fresh samples of the anaerobic ditch sediments revealed the presence of few protozoa. After addition of nutrients to an enrichment culture, the liquid became slightly turbid by the increased growth of bacteria which served as food for the protozoa. During the following few weeks of continued incubation the number of protozoa increased, with a quadri-flagellate as the dominant organism. Monocultures of the latter were obtained by the transfer of cells to the phosphate-buffered FM-medium. Under strictly anaerobic conditions, the flagellates reached a maximal density of 5 x 10^4 cells/ml with doubling times of about 38 h. The cells were subcultured every 3-4 weeks.

Microscopical observations

The flagellate measured 6-8 x 12 μm. The width was variable. Obovate cells sometimes displayed a slightly tapered posterior end (Fig. 1). In well-fed cultures which had reached the stationary growth phase, frequent irregularly shaped giant cells were observed, measuring about 30-40 μm. A set of four flagella is inserted immediately below the anterior apex (Figs. 1-4). The flagella are of equal length, measuring about 1.5 times the cell



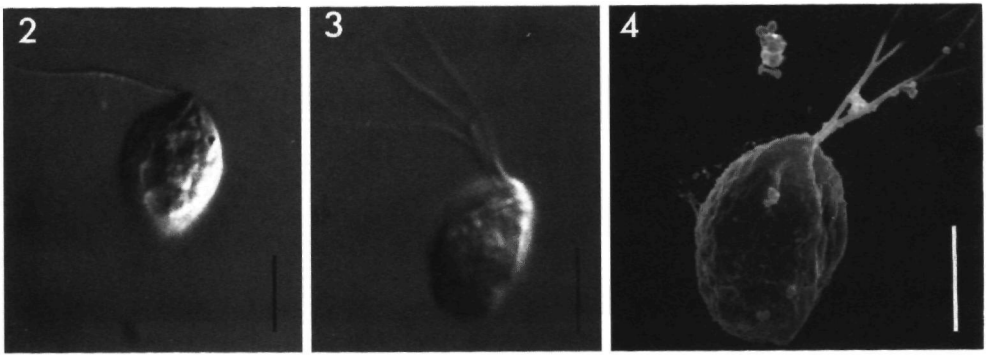


Fig. 1. Diagramic representation of the flagellate cell. a ventral view; b side view

Figs. 2-3. Light micrographs (differential interference contrast) showing the insertion of the four flagella at the apex of the groove (arrow). Scale bar = 5 μm.
Fig. 4. Scanning electron micrograph of the flagellate. The flagella arise subapically at the anterior end of the cell. Scale bar = 5 μm.

length. The ventral groove looks like a slit and extends about 2/3 of the length of the cell(Fig. 1-4). The nucleus was located just below the flagellar insertion. Cysts were ever found. Epifluorescence microscopy revealed the presence of intracellular rod-shaped bacteria showing autofluorescence typical methanogenic bacteria. The number of endosymbionts varied between 12 and 50 per protozoal cell.

Ultrastructural studies confirmed the position of the nucleus in the upper half of the cell

(Fig. 5). The nucleus contained a nucleolus and was associated with the mastigont system by fibrous material. The architecture of the mastigont system was almost identical to that of *Psalteriomonas lanterna*, a heterolobose amoeboflagellate described recently [1-2]. Four kinetosomes are located in the outermost anterior of the cell (Figs. 7, 12). The kinetosomes are arranged in two pairs. To the anterior pair (kinetosomes 1 and 2), a gully-like structure is associated (Figs. 6-9, 11). Striated roots connect the pairs of kinetosomes with the proximal region of a curved microtubule-organizing ribbon (MTOR, see figs. 6, 7, 10). This MTOR is attached to kinetosome 4 and gives rise to two micro-tubular fibres, which support the left and right margins of the groove (Figs. 8-10, 12, 13). The bottom of the groove is occupied by separated, individual microtubules originating from a dense microfibrillar string arising from the MTOR (Figs. 10-13). Rows of microtubules associated with kinetosomes 1 and 3, and microtubules originating from a small MTOR linked to kinetosome 2 (Figs. 7, 9) run along the right and left side of the groove, respectively. A bundle of microfilaments connects the posterior pair of kinetosomes (3 and 4) with the groove-side of the fibre, supporting the right margin of the groove (Figs. 7, 8, 10, 12, 13).

Proximal to the nucleus a spherical aggregate was also found, consisting of clustered hydrogenosome-like organelles and endosymbiotic bacteria (Figs. 5, 15, 16). The latter were rods with pointed ends. The hydrogenosome-like organelles had a double membrane but they showed no infolding of the innermembrane and no association with rough endoplasmic reticulum (Fig. 15). The matrix of these organelles often contained lancunae, but this was probably a fixation artifact. Also, in symbiont-free flagellates, the hydrogenosome-like organelles were aggregated (Fig. 5). In some cultures cells were found in which the cluster of microbodies contained one or two large vacuoles (Fig. 5). The function of these vacuoles is unknown. The food vacuoles were spread throughout the cytoplasm and contained bacteria in various stages of digestion (Fig. 5). No mitochondria or Golgi apparatus was found.

Isolation and characterization of the methanogenic endosymbiont

The procedure of isolation of methanogens in small volumes of liquid medium as described by Goosen et al. [12] was found to be the most suitable method. After two weeks of cultivation, methane production could be measured in the enrichment cultures. After three transfers in liquid mineral medium, supplemented with sodium formate and antibiotics, the cultures were free of contaminating non-methanogenic bacteria. The colonies growing on solid agar medium were circular with an entire margin, convex and whitish yellow. Single cells were slender, non-motile rods, sometimes occurring as chains and forming aggregates in liquid medium. Presence of H_2/CO_2 or formate in the medium was indispensible for growth. No growth was observed on acetate, methanol, methylamine,

glucose or pyruvate under a gas phase of 80% N_2 and 20% CO_2. Growth was observed at temperatures between 15°C to 45°C with an optimum at 35-40°C. In mineral medium with formate, the isolated methanogen showed a generation time of 12-14 h at 37°C.

Although a further characterization of the bacterium is desirable, the above-mentioned properties indicate that most probably the isolate is *Methanobacterium formicicum*. The strain will be referred to as *M. formicicum* strain PS1.

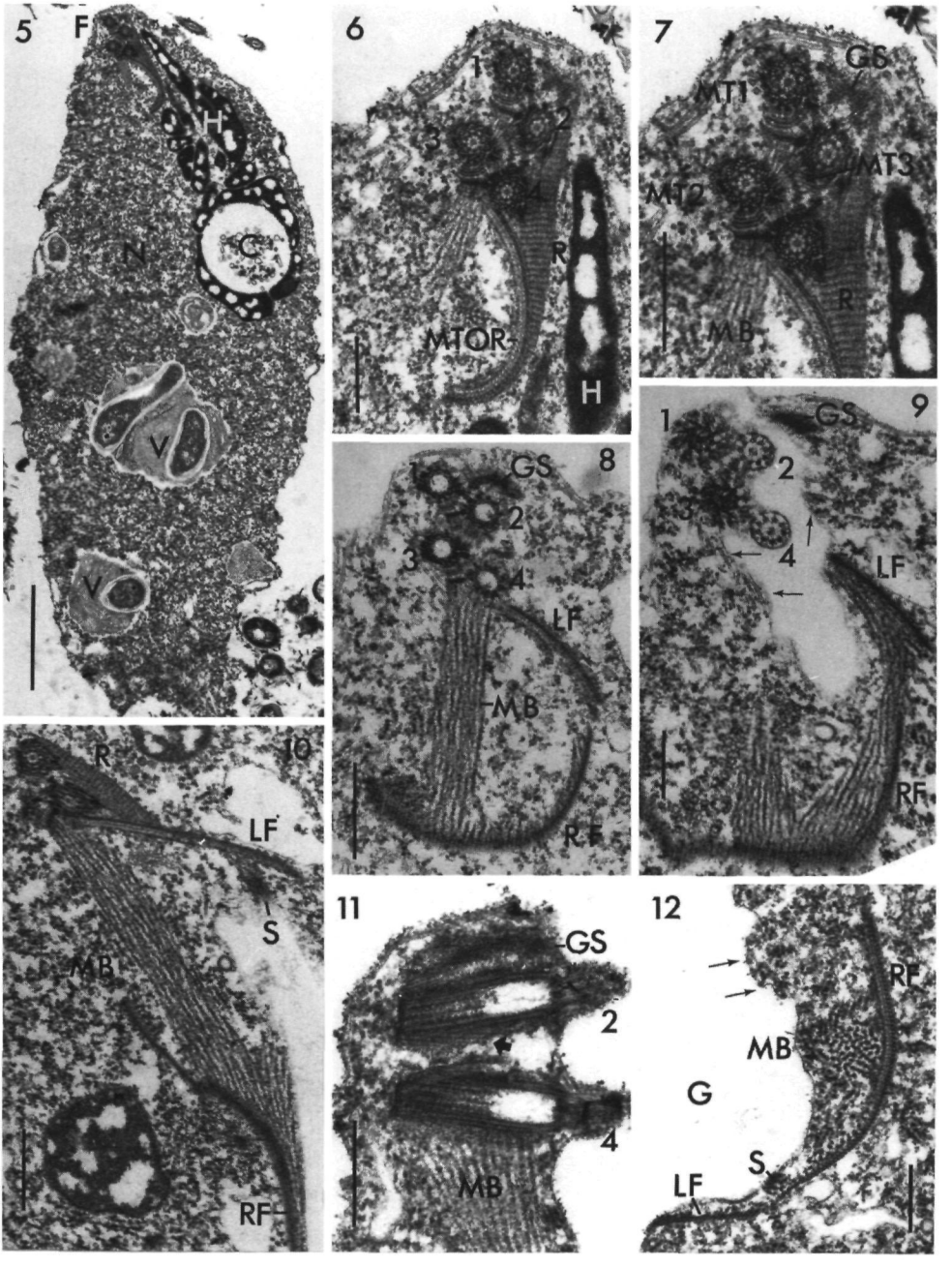
Characterization of the microbodies

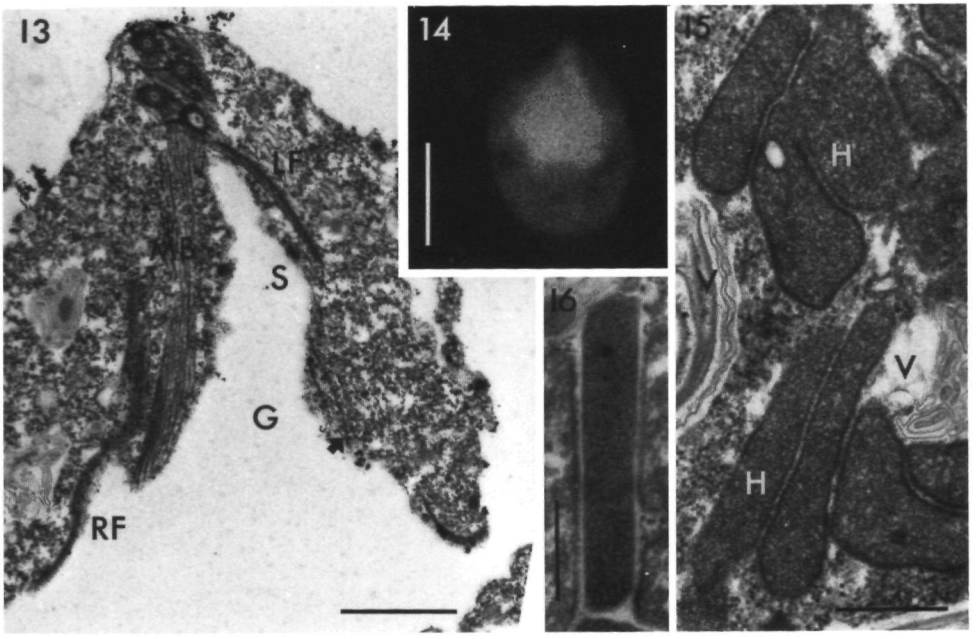
The flagellate showed cross-reaction with the polyclonal hydrogenosomal hydrogenase antiserum, indicating the presence of hydrogenase. The fluorescent staining was localized in the microbodies of the spherical complex in the cell (Fig. 14), identifying them as hydrogenosomes. A cytochemical test for cytochrome oxidase in the flagellate was negative.

Oxygen tolerance

Addition of up to 5% O_2 (v/v) to the headspace of the culture bottles was beneficial for

Figs. 5-12. Transmission electron micrographs of the flagellate.
(5) Longitudinal section of a symbiont-free flagellate cell, showing the general ultrastructural organization. Hydrogenosome-like organelles are clustered together and are located in the vicinity of the nucleus and the mastigont system in the upper half of the cell. Note the large vacuole (C) in the cluster and food vacuoles (v) (scale bar = 2 μm).
(6-9) Transverse sections through the kinetosomes, numbered 1-4. (6) General view of the kinetosomes and the curved ribbon MTOR. A rhizoplast (R) links the pairs of kinetosomes with the MTOR. Scale bar = 0.5 μm. (7) Detail of 6. The kinetosomes are cut at the lower margins and display a cartwheel structure. A microfibrillar bundle (MB) arises from the lamella near kinetsomes 3 and 4. Microtubular rows (MT1, MT2 and MT3) are associated with the kinetosomes. A gully-like structure (GS) is located anteriorly from kinetosome 2. Scale bar = 0.5 μm. (8) Section at a more distal level of the kinetosomes. The microfibrillar bundle connects the kinetosomes 3 and 4 with the posterior region of the MTOR. The MTOR is split into two fibres (RF and LF). Scale bar = 0.5 μm. (9) Section through the transition regions of the flagella, showing the terminal plates in kinetosomes 1 and 3. Microtubules, originating from the microtubular rows MT1, MT2 and MT3, reinforce the margins of the upper end of the groove (arrows). Scale bar = 0.5 μm.
(10) Oblique section, showing the microfibrillar bundle (MB) linking the kinetosomes to the right fibre (RF). A microfibrillar string (S) arises from paracristalline material on the concave side of the MTOR. Scale bar = 0.5 μm.
(11) Longitudinal section through the kinetosomes 2 and 4, showing their position in the outermost end of the cell. The gully-like structure (GS) arises from kinetosome 2 just as the microtubular row MT2 (arrow). Scale bar = 0.5 μm.
(12) Transverse section through the anterior region of the groove. The MTOR is split into two fibres (LF and RF). The microfibrillar string is found between the right fiber and the membrane of the groove. Separated microtubules, originating from MT1 and MT3, reinforce the left side of the groove (arrows). Scale bar = 0.5 μm.
Abbreviations: C = large vacuole; F = mastigont system; G = groove; GS = gully-like structure; H = hydrogenosome; LF = left fibre; MB = micro-fibrillar bundle; MT = microtubular row; MTOR = microtubule-organizing ribbon; R = rhizoplast; RF = right fibre; S = microfibrillar string; V = food vacuole. Bar = 5 μm.


5
F
H
N
C
V
V
6
1
2
3
4
R
MTOR
H
7
GS
MT1
MT2
MT3
R
MB
8
GS
1
2
3
4
LF
MB
RF
9
GS
1
2
3
4
LF
RF
10
R
LF
S
MB
RF
11
GS
2
4
MB
12
RF
MB
G
S
LF



Figs. 13-16. Transmission electron micrographs of the flagellate.
(13) Oblique section through the anterior part of the groove. The margins of the groove are supported by microtubular fibres. The microfibrillar bundle is associated with the groove-side of the right fibre. The string (S) and spaced microtubules (arrow) are shown. Scale bar = 1 μm.
(14) Indirect immunofluorescent staining of fixed flagellate cells. The cells were incubated in rabbit antiserum raised against hydrogenase as described in Material and Methods. The fluorescent stain is localized in the cluster of organelles. Confocal laser scanning micrograph; bar = 5 μm.
(15) Detail of the cluster of hydrogenosomes (H) in a symbiont-free flagellate cell. Scale bar = 0.5 μm.
(16) Methanogenic bacterium (B) in the cluster of hydrogenosomes. Scale bar = 0.5 μm.

Abbreviations: B = methanogenic bacterium; G = groove; H = hydrogenosomes; LF = left fibre; MB = microfibrillar bundle; RF = right fibre; S = micro-fibrillar string; V = food vacuoles.

growth of the flagellates. When 5% O_2 was included in the culture bottles (without refreshment of the gas phase during continued cultivation), an increased growth rate of the flagellate was observed with a doubling time of about 18 h. After the addition of O_2 the medium became quickly opaque, caused by an enhanced growth of micro-aerophilic or

facultatively anaerobic bacteria, which may account for the growth stimulation of the flagellate. In the oxygenated cultures the methane production ceased and methanogenic bacteria could no longer be detected microscopically (Fig. 5). The symbiont-free flagellates obtained in this way died off when brought under strictly anaerobic conditions again. The addition of oxygen did not induce a flagellate-to-amoeba transformation as was observed for *Psalteriomonas lanterna* [1, 2].

By means of the cytochemical test described in Material & Methods, it was shown that superoxide dismutase (SOD) was present in both the cytoplasm and the hydrogenosomes. Catalase, however, was absent in *T. sulcatus*: no bubble formation was observed when a dense cell suspension was added to a drop of 3% aqueous H_2O_2 solution.

DISCUSSION

The ultrastructural organization of the organism described here shows strong similarities with *Psalteriomonas lanterna* (2). Both organisms contain a spherical complex of aggregated bacteria and hydrogenosomes. Secondary and main elements in the mastigont system and the architecture of the groove are identical and justify the placement of the flagellate in the genus *Psalteriomonas*. However, the organism differs from *P. lanterna* in several aspects. The cell shows no fourfold rotational symmetry and the flagellate stage is very stable. Furthermore, the organism lacks the organelles surrounded by rough endoplasmic reticulum as found in *P. lanterna*. We regard the organism as a previously undescribed species and propose the name *Psalteriomonas vulgaris sp. nov.* (*vulgaris*, ordinary).

The general outline of *P. vulgaris* resembles *Tetramitus sulcatus* [15]. However, the latter organism has not been studied by modern techniques. Moreover, *T. sulcatus* has flagella of unequal length and the groove is wider (15). Other quadriflagellates now assigned to the genera *Tetramitus* and *Percolomonas* (e.g. refs. 14, 21) might be psalteriomonads. Study of *Tetramitus descissus* and *T. pyriformis* (G. Brugerolle, unpublished results) revealed a similar ultrastructural identity of *P. lanterna* and *P. vulgaris*, but they lack the cluster of hydrogenosome-like organelles. *Percolomonas* differs from *Tetramitus* and *Psalteriomonas* in having 3 short flagella and a long one (20).

Methanobacterium formicicum seems to be a common endosymbiont in anaerobic freshwater protozoa. The same species has been described for several other taxonomically unrelated organisms [12, 23, 24]. The close association of the bacteria with the microbodies, typified as hydrogenosomes by the immunofluorescence staining, suggests a strong metabolic interrelationship. An interspecies hydrogen transfer seems to be the underlying background for the endosymbiosis [23]. It is suggested that both organisms profit from the

endosymbiosis [22, 23]. The bacterium takes advantage of the location close to the hydrogen source and has no competition with other H_2-utilizing bacteria, e.g. sulfate-reducers [22]. The benefit for the host is more debatable. It is speculated that the protozoan cell profits from the H_2 consumption by the methanogens since H_2 is inhibitory to the host cell metabolism [23, 25]. In laboratory cultures this assumption might be valid as indicated by the inability of symbiont-free flagellates to grow under strictly anaerobic conditions. In this respect the organism differs from other (larger) anaerobic protists depleted of methanogens, which are perfectly capable of living in anaerobic culture [10, 25]. As suggested for *P. lanterna* [4] the culture conditions of the flagellate might be unfavourable for H_2 utilizing bacteria which may explain this discrepancy. However, in their natural environment these inhibitory effects might be minimalized by the low external H_2 tension and the short diffusion pathways [8, 9, 10]. Furthermore, it is suggested that the host might profit from the organic compounds excreted by the methanogenic endosymbiont [8, 9]. Comparative physiological studies of symbiont-free and symbiont-containing flagellates are required to test this assumption for *T. sulcatus*. Another benefit for the host might concern the ability of methanogenic bacteria to fix nitrogen [26].

Anaerobic protozoa vary with respect to O_2 sensitivity [7, 16] *Psalteriomonas vulgaris* tolerates up to 5% O_2. However, the oxygen was added once and may drop quickly to lower concentration due to the O_2 consumption by the food bacteria and, possibly, by the flagellates. Cultivation under a gas phase with constant composition indicated that *P. vulgaris* tolerates only $pO_2 \leq 1\%$ (C. Broers, unpublished results). The aerotolerance of the organisms might depend on the presence of superoxide dismutase (SOD) and not on the presence of catalase. SOD was found to be present in the hydrogenosomes, and under natural conditions it may protect the oxygen-sensitive hydrogenase and probably other hydrogenosomal enzymes. It remains unknown, if the hydrogenosomes are active under the elevated O_2 tensions. However, the hydrogenosomal functions may be dispensible since cultivation of trichomonads devoid of hydrogenosomes has been reported [18]. On the other hand, O_2 may replace protons as terminal electron acceptors, thus stimulating the hydrogenosomal metabolism [18].

Unlike in *P. vulgaris*, the endosymbiotic methanogens in some anaerobic ciliates remain viable, even after prolonged exposure to elevated levels of O_2 [7]. Under an atmosphere of 5% O_2 the methanogens in the ciliates may become inactivated [7], however, in the giant amoeboflagellate *Pelomyxa palustris* the endosymbionts continue to produce methane [24]. A protection against oxygen toxicity by diffusion limitation is suggested for these protozoa [7, 26]. However, *P. vulgaris* is much smaller than the above-mentioned protozoa, and the size of the flagellate is probably insufficient to protect the endosymbionts on exposure to lethal concentrations of O_2.

P. vulgaris is a welcome addition for comparative physiological and biochemical studies on heterolobose amoeboflagellates, especially with regard to the type of redox organelles present in these organisms. *P. vulgaris* has hydrogenosomes, but the organism is related to aerobic amoeboflagellates such as *Tetramitus rostratus* and *Naegleria* which have mitochondria. An intermediate position could be occupied by *P. lanterna* which presumably has both mitochondria and hydrogenosomes [1, 2].

Taxonomic summary

Psalteriomonas vulgaris n. spec.

Diagnosis. Mononucleated flagellate cell, 10-12 μm long. Groove extends half to full body length. Four flagella of equal length are inserted in the apex of the groove, subapically at the outermost anterior end of the cell. Amoeboid form uncertain; cyst stage unknown. Clustered hydrogenosome-like organelles and methanogenic endosymbionts are present.

Ultrastructural organization: as for genus.

REFERENCES

1. Broers C.A.M., Stumm C.K and Vogels G.D. (1989): A heterolobose amoeboflagellate associated with methanogenic bacteria. In: Lloyd D., Coombs G.H. & Paget T.A. (eds.): Biochemistry and Molecular Biology of "Anaerobic" Protozoa, pp 22-31. Harwood publishers, London.
2. Broers C.A.M., Brugerolle G., Stumm C.K. and Vogels G.D. (1990): *Psalteriomonas lanterna* gen. nov. sp. nov., a free-living amoeboflagellate isolated from freshwater anaerobic sediments. Eur. J. Protistol., *25*, 369-380.
3. Broers C.A.M., Stumm C.K. and Vogels G.D. (1991): Axenic cultivation of the anaerobic free-living ciliate *Trimyema compressum*. J. Protozool., *38*, 507-511.
4. Broers C.A.M., Berkhout R.J.M., Lin Y.HY, Stumm C.K. and Vogels G.D. (1992): Oxygen responses of the free-living anaerobic amoeboflagellate *Psalteriomonas lanterna*. FEMS Microbiol. Ecol., in press.
5. Burnstone M.S. (1961): Modifications of histochemical techniques for the demon stration of cytochrome oxidase. J. Histochem. Cytochem., *9*, 59-65.
6. Doddema H.J. and Vogels G.d. (1978): Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol., *36*, 752-754.
7. Fenchel T. and Finlay B.J. (1990): Oxygen toxicity, respiration and behavioural responses to oxygen in free-living anaerobic ciliates. J. Gen. Microbiol., *136*, 1953-1959.
8. Fenchel T. and Finlay B.J. (1991a): Endosymbiotic methanogenic bacteria in anaerobic ciliates: significance for the growth efficiency of the host. J. Protozool., *38*, 18-22.
9. Fenchel T. and Finlay B.J. (1991b): Synchronous division of an endosymbiotic methanogenic bacterium in the anaerobic ciliate *Plagiopyla frontata* Kahl. J. Proto zool., *38*, 22-28.
10. Fenchel T. and Finlay B.J. (1991c): The biology of free-living anaerobic ciliates.

Eur. J. Protistol., *26*, 201-215.
11. Finlay B.J. and Fenchel T. (1989): Hydrogenosomes in some anaerobic protozoa resemble mitochondria. FEMS Microbiol. Lett., *65*, 311-314.
12. Goosen N.K., Horemans A.M.C., Hillebrand S.J., Stumm C.K. and Vogels G.D. (1988): Cultivation of the sapropelic ciliate *Plagiopyla nasuta* Stein and isolation of the endosymbiont Methanobacterium formicicum. Arch. Microbiol., *150*, 165-170.
13. Goosen N.K., Wagener S. and Stumm C.K. (1990): A comparison of two strains of the anaerobic ciliate *Trimyema compressum*. Arch. Microbiol., *153*, 187-192.
14. Larsen J. and Patterson D.J. (1990): Some flagellates (Protista) from tropical marine sediments. J. Nat. History, *24*, 801-937.
15. Lemmermann E. (1914): Flagellatae, I, In: Pascher A. (ed.) Die Süsswasserflora Deutschlands, Österreichs und der Schweiz, pp. 43-46. Fischer, Jena.
16. Mack S.R. and Müller M. (1978): Effect of oxygen and carbon dioxide on the growth of *Trichomonas vaginalis* and *Tritrichomonas foetus*. J. Parasitol., *64*, 927-929.
17. McInerney M.J., Bryant M.P. and Pfennig N. (1979): Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. Arch. Microbiol., *122*, 129-135.
18. Müller M. (1988): Energy metabolism of protozoa without mitochondria. Ann. Rev. Microbiol., *42*, 465-488.
19. Parducz B. (1967): Ciliary movement and coordination in ciliates. Int. Rev. Cyt., *21*, 91-128.
20. Patterson D.J. and Zölffel (1991): Heterotrophic flagellates of uncertain taxonomic position. In: Patterson D.J. and Larsen J. (eds.): The Biology of free-living heterot rophic flagellates. Systematic Association Special Volume *45*, pp. 427-475. Clarendon Press, Oxford.
21. Skvortzov B.V. (1974): On some colourless flagellates from Java and Brasil. Reinwardtia, *9*, 177-182.
22. Stumm C.K. and Zwart K.B. (1986): Symbiosis of protozoa with hydrogen utilizing methanogens. Microbiol. Sci., *3*, 100-105.
23. Van Bruggen J.J.A., Zwart K.B., Van Assema R., Stumm C.K. and Vogels G.D. (1984): *Methanobacterium formicicum*, an endosymbiont of the anaerobic ciliate *Metopus striatus* McMurrich. Arch. Microbiol., *139*, 1-7.
24. Van Bruggen J.J.A., Van Rens G.L.M., Geertman E.J.M., Zwart K.B., Stumm C.K. and Vogels G.D. (1988): Isolation of a methanogenic endosymbiont of the sapropelic amoeba *Pelomyxa palustris* Greeff. J. Protozool., *35*, 20-23.
25. Wagener S. and Pfennig N. (1987): Monoxenic culture of the anaerobic ciliate *Trimyema compressum* Lackey. Arch. Microbiol., *144*, 367-374.
26. Whitman W.B. (1985): Methanogenic bacteria. In: Woese C.R. and Wolfe R.S. (eds.): The Bacteria, Vol. 8, pp. 3-84. Academic Press, New York.
27. Zwart K.B., Goosen N.K., Van Schijndel M.W., Broers C.A.M., Stumm C.K. and Vogels G.D. (1988): Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis*, *Plagiopyla nasuta* and *Trimyema compres sum*. J. Gen. Microbiol., *134*, 2165-2170.

CHAPTER 6

AN ELECTROMIGRATION TECHNIQUE,APPLIED FOR THE CONCENTRATION OF ANAEROBIC PROTOZOA FROM MASS CULTURES

Journal of Microbiological Methods 14 (1992) 217–220

MIMET 00465

An electromigration technique, applied for the concentration of anaerobic protozoa from mass cultures

Cees A. M. Broers, Henri O. F. Molhuizen, Claudius K. Stumm and Godfried D. Vogels

Department of Microbiology, Faculty of Science, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands

(Received 6 February 1991; revision received 21 September 1991; accepted 27 September 1991)

Summary

For rapid concentration of protozoan cells from anaerobic mass cultures and separation from food bacteria and debris, an electromigration apparatus was developed. Anaerobic protozoa from 3-l cultures could be concentrated into a few milliliters within 1 h, while anoxic conditions were maintained. The method was tested with cultures of the amoeboflagellate *Psalteriomonas lanterna* and the ciliate *Trimyema compressum*.

Key words: Anaerobic protozoa; Electromigration; *Psalteriomonas*; *Trimyema*

Introduction

Cultivation of anaerobic free-living protozoa has made considerable progress [1–4]. Research on the physiology and biochemistry of these organisms, however, has been hampered by the difficulties in obtaining high cell densities and in separating the protozoa from accompanying food bacteria. Harvesting of the protozoa by gentle centrifugation, followed by a repeated washing of the sediment to remove the bacteria, is often unsuccessful due to the presence of aggregated bacteria in the medium which sediment together with the cells. Also centrifugal elutriation [5], a strong tool to separate particles in size and density, does not give satisfactory results in this case. A better method was found in the electromigration technique [6, 7]. In this paper an

Correspondence to: C. A. M. Broers, Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands.

electromigration apparatus is described which allows the rapid isolation and concentration of anaerobic protozoa from mass cultures.

Materials and Methods

Organisms

Two anaerobic free-living protozoa were used to test the electromigration apparatus, viz., the ciliate *Trimyema compressum* and the amoeboflagellate *Psalteriomonas lanterna*. Both organisms were originally isolated from a sedimentation pond of a waste water treatment plant near Nijmegen [1, 8]. *T. compressum* was cultivated monoxenically with *Bacteroides* sp. strain WoCb 15 as previously described [3, 4].

Symbiont-free cells of *P. lanterna* were grown xenically in the medium described earlier [1] with 1% O_2 in the headspace of culture bottles. To obtain maximum growth, the gas phase was flushed two times a week with N_2/O_2 (99%/1%).

Electromigration apparatus

To harvest the cells, a modified electromigration apparatus after Van Wagtendonk [6, 7] was used, as shown in Fig. 1. The cells migrate from the large compartment A, through tube B into the small collection chamber C. Chamber C and tube B (length 135 mm, i.d. 18 mm) are filled with electromigration medium (10 mM Na/K phosphate buffer plus 0.2 mM cysteine HCl, pH 6.8). This medium was first made anaerobic by evacuation three times for 4 min and gassing with O_2-free N_2.

During flushing with N_2, via stopcock F, chamber A was filled with the cell culture. To avoid mixing of culture medium and electromigration medium stopcock D is closed. Chambers A and C are separated from direct contact with the platinum electrodes by KCl agar bridges (3% KCl, 3% agar). Agar bridge 2 is joined to chamber C with a screw cap.

Migration starts by opening stopcock D. The current used in our experiments was 1 mA and a voltage of 60 V (power supply: E350, Farnell Instruments, Wetherby, UK). The optimal current and voltage had previously been estimated with the aid of a small electromigration device described by Wagener et al. [9]. The migrations were run at room temperature. Concentrated cells from chamber C were gained by opening stopcock E and F.

Results and Discussion

Electromigration techniques are described to concentrate suspensions of ciliates relatively free from bacteria and debris [6, 10, 11] and for sterilization of *Paramecium* to start axenic cultures [12]. Wagener et al. [9] used electromigration to extract

TABLE 1

Organism	Cell density compartment A (cells · ml^{-1})	Migration time (min)	Yield (number of cells)	Recovery (%)
P. lanterna	$8 - 11 \cdot 10^3$	90	$20 - 28 \cdot 10^6$	75 – 95
T. compressum	$0.7 - 1.2 \cdot 10^3$	45	$2 - 3.5 \cdot 10^6$	95

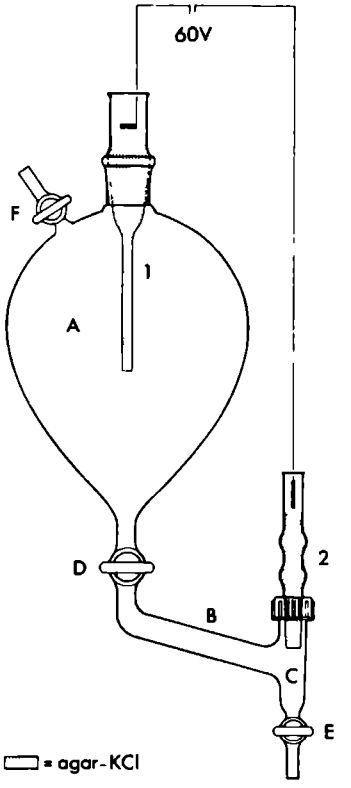


Fig 1. Electromigration apparatus. Electrodes are in contact with KCl agar bridges 1 and 2. For further details, see text.

anaerobic ciliates from sludge samples on a small scale. However, their experiments were performed without precautions to prevent oxygenation of the medium, which may have influenced the recovery and viability of those ciliates. Own experiments showed that oxygenation of the medium lead to a rapid loss of motility of *T. compressum* and *P. lanterna*. The electromigration apparatus and the use of KCl agar bridges described here facilitate the performance of the experiments under anoxic conditions.

Both *T. compressum* and the flagellate stage of *P. lanterna* showed galvanotaxis and swam to the cathode. A voltage of 50 – 60 V and a current of 1 mA was found to be optimal for both organisms. Cultures in an early stationary phase gave the best results. As appeared from preliminary experiments, the enrichment of the protozoa was improved by a slight slope of tube B (15° from horizontal). Within 90 min the migration of a 3-l flagellate suspension was completed. After the procedure 75 – 95% of the *P. lanterna* cells were concentrated in compartment C. For *T. compressum* the migration time was even shorter. Within 45 min ≈95% of the cells were found in compartment C. The same efficiency of recovery was found for *Paramecium* by Van Wagtendonk [7]. However, the migration time of *Paramecium* was longer, possibly due to the longer distance the ciliates had to swim. In our apparatus the length of the migration path was found to be sufficient to get rid of debris and bacteria. The numbers of accompanying bacteria in compartment C were negligible. Plate counting

indicated that < 1000 bacteria $\cdot ml^{-1}$ were present in the protozoa samples During the migration process the cells settled in the collection chamber C, just above stopcock E. This enabled us to obtain 1 5 – 2 ml of concentrated cell suspensions. The electromigration technique has proved to be an excellent tool for concentrating and purifying anaerobic protozoa, suited to facilitate biochemical study on those organisms.

References

1 Broers, C A M , Brugerolle, G , Stumm, C K and Vogels, G D (1990) *Psalteriomonas lanterna* gen nov , sp nov , a free living amoeboflagellate isolated from freshwater anaerobic sediments Eur J Protistol 25, 369 – 380

2 Finlay, B J and Fenchel, T (1989) Hydrogenosomes in some anaerobic protozoa resemble mitochon dria FEMS Microbiol Lett 65, 311 – 314

3 Goosen, N K , Wagener, S and Stumm, C K (1990) A comparison of two strains of the anaerobic ciliate *Trimyema compressum* Lackey Arch Microbiol 149, 4 – 11

4 Wagener, S and Pfennig, N (1987) Monoxenic culture of the anaerobic ciliate *Trimyema compres sum* Lackey Arch Microbiol 149, 4 – 11

5 Plas, A , De Witte, T , Wessels, J and Haanen, C (1988) A new multichamber counterflow centrifu gation rotor with high separation capacity and versatile potentials Exp Hematol 16, 355

6 Van Wagtendonk, W J , Simonsen, D H and Zill, L P (1952) The use of electromigration tech niques in washing and concentrating cultures of *Paramecium aurelia* Phys Zool 25, 312 – 317

7 Van Wagtendonk, W J and Soldo, A T (1970) Methods used in the axenic cultivation of *Parameci um aurelia* In Method of Cell Physiology IV (Prescott, D M , ed), pp 117 – 130, Academic Press, New York

8 Zwart, K B , Goosen, N K , Van Schijndel, M W , Broers, C A M , Stumm, C K and Vogels, G D (1988) Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis, Plagiopyla nasuta* and *Trimyema compressum* J Gen Microbiol 134, 2165 2170

9 Wagener, S , Stumm, C K and Vogels, G D (1986) Electromigration, a tool for studies on anaerobic ciliates FEMS Microbiol Ecol 38, 197 203

10 Borkott, H (1975) A method for quantitative isolation and preparation of particle free suspensions of bacteriophagous ciliates from different substrates for electronic counting Arch Protistenk 117, 261 268

11 Schmidt, H J (1982) New methods for cultivating, harvesting and purifying mass cultures of the hypotrich ciliate *Euplotes aediculatus* J Protozool 29, 132 – 135

12 Van Wagtendonk, W J and Hackett, P I (1949) The culture of *Paramecium aurelia* in the absence of other living organisms Proc Nat Acad Sci 35, 155 – 159

SUMMARY

Anaerobic protists live exclusively in anoxic environments and they perform fermentative metabolism. Many anaerobic protists posses hydrogenosomes, organelles regarded as the anaerobic equivalents of mitochondria. These organelles are the site of a substrate level phosphorylation and they produce hydrogen. Most free-living anaerobic protists harbour methanogenic endosymbionts that presumably convert the produced hydrogen into methane. In chapter 1 it is shown that anaerobic protists have a polyphyletic origin. Apart from their energy metabolism and symbiosis with methanogens, flagellated protists are of interest because they may provide valuable information concerning early eukaryotic evolution and organelle symbiosis.

In chapter 2 a free-living anaerobic amoeboflagellate is extensively described which belongs to the class Heterolobosea. The flagellate stage showed a fourfold rotational symmetry with four nuclei, four mastigont systems and four grooves. The organism was recognized as a new genus and the name *Psalteriomonas lanterna* is proposed. In *P. lanterna* a maximal grade of association between methanogenic endosymbionts and hydrogenosome-like microbodies has been reached, suggesting a strong metabolic interrelationship. They form a distinct aggregate in the centre of the cell. Besides these presumed hydrogenosomes, cytoplasmic organelles were found, which were surrounded by rough endoplasmic reticulum, a characteristic of the mitochondria of heterolobosean amoeboflagellates. However, the organelles have no clear cristae and they lack cytochrome oxidase.

The amoeboflagellate did not tolerate high oxygen tensions (Chapter 3). Low oxygen concentrations stimulated the growth of the flagellate, probably due to to increased growth of food bacteria. In these cultures the flagellates tended to transform to a mononucleated amoeboid form. The methanogenic bacteria were lost even under microaerobic conditions and cells deprived of methanogens did not grow when they were returned to anaerobic conditions. The methanogen probably protects the cells against high intracellular pH_2.

In chapter 4 the requirement for H_2 of the methanogenic endosymbiont was shown. The bacterium was isolated and identified as *Methanobacterium formicicum*. A monoxenic culture of the flagellate was established, which means a step forward in studying the anaerobic metabolism of the organism.

By using a cytochemical staining method, hydrogenase could be localized in the microbodies, identifying them as hydrogenosomes (chapter 5). This finding was confirmed by an immunocytochemical study using antibodies against hydrogenosomal hydrogenase. The presumed mitochondria could be selectively stained with rhodamine 123, a dye specific for mitochondria, and the organelles probably contain DNA. However, cytochromes could not be detected and cytochemical tests for succinate dehydrogenase and citrate (isocitrate) hydro-lyase were negative. The evolutionarty significance of the presence of both organelles is discussed. It seems that the hydrogenosomes have a symbiotic origin from an anaerobic prokaryote. Futhermore some evolutionary aspects concerning the organism itself are discussed.

In chapter 6 a related amoeboflagellate, *Psalteriomonas vulgaris*, n. spec., is studied. The ultrastructural organization of the mastigont system and the groove is almost identical to that of *P. lanterna*. The organism differs from *P. Lanterna* by the absence of modified mitochondria, the stability of the flagellate stage and by the absence of a tetranucleated stage. Just as *P. lanterna*, the organism has a cluster of microbodies and methanogenic endosymbionts. The microbodies could be identified as hydrogenosomes using a immunocytochemical staining technique. The methanogenic endosymbiont could be isolated and identified as *Methanobacterium formicicum*.

In chapter 7 an electromigration technique is described which enables us to purify anaerobic protists from mass cultures. Anaerobic conditions are maintained and cells from 3 liter cultures could be concentrated to a final volume of 1.5 ml.

SAMENVATTING

Protisten zijn eencellige eukaryoten die meestal alleen met behulp van een microscoop zijn waar te nemen. De verscheidenheid aan soorten binnen deze groep van organismen is enorm en sommige protisten zijn in staat om in zuurstofloze milieus te leven. Deze anaerobe protisten missen mitochondriën en bedrijven een fermentatief metabolisme (Hoofdstuk 1). Veel van deze organismen bezitten hydrogenosomen, organellen die energie produceren waarbij waterstof vrijkomt. De meeste vrijlevende anaerobe protisten huisvesten bovendien endosymbiotische methaanbacteriën, die de geproduceerde waterstof omzetten in methaan. De anaerobe protisten hebben alleen hun habitat gemeen; de diverse groepen organismen zijn niet aan elkaar verwant. De vrijlevende anaerobe flagellaten zijn bijzonder interessant. Studie aan deze organismen kan niet alleen belangrijke informatie opleveren betreffende gastheer-endosymbiont interactie, maar ook waardevolle gegevens over vroege eukaryote evolutie en het ontstaan van organellen.

In hoofdstuk 2 wordt een vrijlevende anaerobe amoeboflagellaat uitgebreid beschreven, die tot de klasse Heterolobosea behoort. De flagellaat vertoont een vierzijdige radiaire symmetrie met vier kernen, vier mastigontsystemen en vier orale groeven. Het organisme behoort tot een nieuw geslacht en de naam *Psalteriomonas lanterna* wordt voorgesteld. In *P. lanterna* is een maximale graad van associatie tussen methanogene endosymbionten en hydrogenosoomachtige organellen bereikt, hetgeen een sterke metabole interactie suggereert. Beide componenten vormen een bolvormig aggregaat in het midden van de cel. Naast deze organellen is er nog een tweede type organel in het cytoplasma aanwezig. Deze vrijliggende organellen zijn omringd door ruw endoplasmatisch reticulum, een eigenschap die kenmerkend is voor de mitochondriën van heterolobose amoeboflagellaten. Echter, de organellen missen duidelijke cristae en zij bezitten geen cytochroomoxidase.

In hoofdstuk 3 wordt aangetoond dat *P. lanterna* geen hoge zuurstofconcentraties verdraagt. Lage concentraties zuurstof stimuleert de groei van de flagellaat, dat waarschijnlijk veroorzaakt wordt door de toegenomen groei van voedselbacteriën. Bovendien trad er in deze cultures een transformatie naar een eenkernig amoeboïd stadium op. De amoeboflagellaat verliest zijn

methanogene endosymbionten zelfs onder micro-aerobe omstandigheden en de endosymbiontvrije cellen zijn niet meer in staat om onder anaerobe condities te groeien. De methanogene endosymbiont beschermt de gastheercel mogelijk tegen te hoge intracellulaire concentraties waterstof.

In hoofdstuk 4 blijkt dat de methanogene endosymbiont H_2 nodig heeft voor groei. De bacterie werd geïsoleerd en geïdentificeerd als *Methanobacterium formicicum*. De flagellaat werd in monoxene cultuur gebracht met *Pseudomonas fluorescens* als voedselbacterie. Dit betekent een belangrijke stap voorwaarts in het bestuderen van het anaerobe metabolisme van het organisme.

De organellen van het centrale aggregaat konden geïdentificeerd worden als hydrogenosomen door de lokalisatie van het enzym hydrogenase (hoofdstuk 5). De veronderstelde mitochondriën namen rhodamine 123 op, een kleurstof specifiek voor mitochondriën, en zij bevatten mogelijk DNA. Echter, er konden geen cytochromen aangetoond worden, evenmin als de enzymen succinaat dehydrogenase en citraat (isocitraat) hydro-lyase. De evolutionaire betekenis van de aanwezigheid van beide organellen wordt bediscussieerd. Mogelijk zijn de hydrogenosomen uit een anaerobe bacterie geëvolueerd. Verder worden enkele evolutionaire aspecten betreffende de amoeboflagellaat zelf besproken.

In hoofdstuk 6 wordt nog een Psalteriomonade amoeboflagellaat, *P. vulgaris*, bestudeerd. De Ultrastructurele organisatie van het mastigontsysteem en de ventrale groeve is nagenoeg identiek aan die van *P. lanterna*. Het organisme verschilt van *P. lanterna* door de afwezigheid van gemodificeerde mitochondriën en een vierkernig stadium. Bovendien is het flagellatenstadium erg stabiel. Net als *P. lanterna* heeft het organisme een cluster van hydrogenosomen en methaanbacteriën. De endosymbiont kon geïsoleerd en geïdentificeerd worden als *M. Formicicum*.

In hoofdstuk 7 wordt een electromigratietechniek beschreven, die ons in staat stelt om anaerobe protisten van massacultures te concentreren tot een volume van ca. 1,5 ml. Anaerobe condities zijn gewaarborgd en de cellen worden goed gescheiden van debris en voedselbacteriën.

PUBLICATIONS

Mariman E.C.M., Broers C.A.M., Claesen C.A.A., Tesser G.I. and Wieringa B. (1987). Structure and function of the human creatine kinase B gene. Genomics *1*, 126-137.

Zwart K.B., Goosen N.K., Van Schijndel M.W., Broers C.A.M., Stumm C.K., and Vogels G.D. (1988). Cytochemical localization of hydrogenase activity in the anaerobic protozoa *Trichomonas vaginalis, Plagiopyla nasuta* and *Trimyema compressum*. J. Gen. Microbiol. *134*, 2165-2170.

Broers C.A.M., Goosen N.K., Stumm C.K. and Vogels G.D. (1989). Endosymbiosis of methanogenic bacteria in a sapropelic flagellate. J. Protozool. *36*, 2A. (Abstract).

Broers C.A.M., Stumm C.K. and Vogels G.D. (1989). A heterolobose amoeboflagellate associated with methanogenic bacteria. In: Lloyd D., Coombs G.H. and Paget T.A. (eds), Biochemistry and molecular Biology of "Anaerobic" Protozoa. Harwood Academic Publishers. Chur (Zw), pp 22-31.

Broers C.A.M., Stumm C.K. and Vogels G.D. (1989). A sapropelic amoeboflagellate with hydrogenosomes and mitochondrion-like organelles. Forum Microbiol. *12* 53. (Abstract).

Broers C.A.M., Brugerolle G. Stumm C.K. and Vogels G.D. (1990). *Psalteriomonas lanterna* gen. nov., spec. nov., a free-living amoeboflagellate isolated from freshwater anaerobic sediments. Eur. J. Protist. *25*, 369-380.

Goosen N.K., Broers C.A.M., Hombergen E.J., Stumm C.K. and Vogels G.D. (1990). Effect of fixation on activity and cytochemistry of hydrogenosomal enzymes in *Trichomonas vaginalis*. J. Gen. Microbiol. *136*, 2189-2193.

Gijzen H.J., Broers C.A.M. Barughare M. and Stumm C.K. (1991). Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in cockroach hindgut. Appl. Environm. Microbiol. *57*, 1630-1634.

Broers C.A.M., Stumm C.K. and Vogels G.D. (1991). Axenic cultivation of the anaerobic ciliate *Trimyema compressum*. J. Protozool. *38*, 507-511.

Broers C.A.M., Molhuizen H.O.F., Stumm C.K. and Vogels G.D. (1992). An electromigration technique, applied for the concentration on anaerobic protozoa from mass cultures. J. Microbiol. Meth. *14*, 217-220.

Broers C.A.M., Stumm C.K. and Vogels G.D. (1992). Monoxenic cultivation of the anaerobic amoeboflagellate *Psalteriomonas lanterna* and isolation of the methanogenic endosymbiont. FEMS Microbiol. lett. *92*, 115-118.

Broers C.A.M. Meijers H.H.M. Symens J.C., Brugerolle G., Stumm C.K. and Vogels G.D. (1992). Symbiotic association of *Psalteriomonas vulgaris* n. spec. with *Methanobacterium formicicum*. Eur. J. Protistol. Submitted.

Broers C.A.M., Berkhout R.J.M., Yu Hua Lin, Stumm C.K. and Vogels G.D. (1992). Oxygen responses of the free-living anaerobic amoeboflagellate *Psalteriomonas lanterna*. FEMS Microbiol. Ecol. In press.

Broers C.A.M., Van De Wiel M.J.A., Stumm C.K. and Vogels G.D. (1992). Hydrogenosomes and presumed mitochondria in the anaerobic amoeboflagellate *Psalteriomonas lanterna*. BioSystems. Submitted.

DANKWOORD

Dit proefschrift is het resultaat van onderzoek in teamverband. Alle mensen die een wezenlijke bijdrage hebben geleverd bij het tot stand komen van dit proefschrift wil ik dan ook bedanken.
Allereerst gaat mijn dank uit naar de doctoraal studenten die een belangrijk deel van het praktische werk voor hun rekening hebben genomen: Richard Sinke, Moniek Jansen, Ron Berkhout, Niek Appeldoorn, Marja Willems, Henry Molhuizen, Erik-Jan Hombergen, Joep Symens, Rianne van de Wiel en Henk Meijers. Apart wil ik Yu Hua Lin vermelden, die veel belangrijk kweekwerk heeft verricht. Elma de Laat ben ik dankbaar voor het typen van dit manuscript. Voorts wil ik alle (oud)collega's van de afdeling Microbiologie, waarmee ik gedurende het onderzoek prettig heb samengewerkt, bedanken.
Prof. H. Tabak (AMC) ben ik erkentelijk voor het interpreteren van de cytochroomspectra en het beschikbaar stellen van mitochondriele DNA-probes.
Prof. Bé Wieringa, Letty Lubsen en prof. J. Schoenmakers wil ik bedanken voor adviezen en voor de verleende gastvrijheid om op hun laboratoria DNA-onderzoek te verrichten. Jammer dat de resultaten betreffende het mitochondriele DNA niet in het proefschrift zijn opgenomen.
I would like to thank Guy Brugerolle for his hospitality during my visit to his laboratory and for the fruitful collaboration. Futhermore, I would like to express my thanks to Miklos Müller and Donald Lindmark for advice.
Ook ben ik de medewerkers van de afdelingen Illustratie dankbaar voor de vervaardiging van respectievelijk tekeningen en grafieken en de verschillende typen elektromigratie-apparaten.
Tot slot wilde ik mijn ouders, Shelley en onze kinderen, Arabella en Alfonso, bedanken voor de steun die zij mij hebben gegeven.

CURRICULUM VITAE

Cees Broers werd op 30 oktober 1954 geboren te Tilburg. Na ULO en HAVO, werd in 1979 de tweede graads onderwijsbevoegdheid voor biologie en aardrijkskunde behaald aan het Mollerinstituut te Tilburg. Na enkele jaren werkzaam te zijn geweest in het onderwijs, werd in 1982 de biologiestudie voortgezet aan de Katholieke Universiteit van Nijmegen. Het doctoraalprogramma omvatte een bijvak Chemische Cytologie (Dr. A. Pieck; weefselkweek en onderzoek aan de ADP-ribosylatie van nucleaire lamina) en Antropogenetica (Prof. dr. B. Wieringa; isolatie van het creatine kinase B gen) en het hoofdvak microbiologie (Prof. dr. ir. G.D. Vogels en dr. K.B. Zwart; cytochemische lokalisatie van hydrogenase in anaerobe protisten). Het doctoraal examen werd in februari 1986 afgelegd.

Van februari 1986 tot september 1990 was hij als wetenschappelijk medewerker werkzaam op het Laboratorium voor Microbiologie van de Katholieke Universiteit te Nijmegen.

Posters werden gepresenteerd bij de jaarlijkse bijeenkomsten van de Nederlandse Vereniging Voor Microbiologie, de "6th European Conference on Ciliate Biology" augustus 1987 te Aarhus, Denemarken, "41st Annual Meeting of the Society of Protozoologists", juli 1988 te Bristol, U.K., "8. Wissenschaftliche Tagung der Deutschen Gesellschaft für Protozoologie", maart 1989, te Iserlohn. Voordrachten zijn gehouden tijdens het "Symposium on Anaerobic Protozoa", juli 1988, te Cardiff, U.K., de "Gemeinsame Tagung der VAAM, DGHM und NVvM", maart 1989, Marburg, Duitsland, "10. Wissenschaftliche Tagung der Deutschen Gesellschaft für Protozoologie", maart 1991, te Giessen, "119th Meeting of the Society for General Microbiology", april 1991, Edinburgh, U.K.

Vanaf 1 september 1991 is hij werkzaam aan de afdeling Experimentele Dierkunde van de Katholieke Universiteit van Nijmegen.

Hij is gehuwd en heeft twee kinderen.